standards. Although the selection of a given RNA standard depends primarily on the purpose and application, all RNA standards should be tested for a clearly defined number of copies of a given sequence within an RNA preparation over some linear range (Cronin et al. 2004).

Some initiatives are raising awareness of the effects of variables that might hamper data comparability and are working toward developing best practice guidelines for microarray-based measurements (Hopkins et al. 2004). For example, recommendations for best practice in array normalization, together with performance characteristics in terms of sensitivity, accuracy, and comparability of different array platforms (cDNA and oligo, spotted and *in situ* synthesis), are beginning to emerge together with proposals for transparency and availability through publicly accessible databases (http://www.vam.org.uk). Other initiatives are considering the use of quality metrics for standardizing and validating array-based toxicogenomics measurements. The extent to which such efforts will be pursued and the impact they will have upon the standardization issues that are a necessary prerequisite to the validation exercises remain to be seen.

Quality assurance and Good Laboratory Practice. GLP is intended to promote proper documentation, quality, and authenticity of toxicity test data and is required for data acceptance by regulatory agencies (e.g., U.S. FDA, U.S. EPA). At the international level, GLP has been promulgated under the OECD guidelines program (OECD 1998). As part of the progression toward regulatory acceptance, toxicogenomics experiments should ideally be conducted in accordance with GLP. However, at present, most large-scale toxicogenomics efforts are not arising from GLP-compliant laboratories, and requiring compliance for data submission could greatly hamper the technical advancement of new technologies and retard their migration into the regulatory arena. To avoid discouraging technologic progress while maintaining a level of GLP conformity, it could be argued that for research and technical development and improvement purposes, it might be acceptable if array-based studies could at least measure up to the reporting standards required by GLP. However, with the adoption of the toxicogenomics-based technologies into regulatory decision-making practices, GLP compliance undoubtedly will be expected. Procedural aspects of GLP compliance not currently captured in MIAME-Tox (minimum information about a microarray experiment for toxicogenomics) will need to be identified but can be incorporated over time. Until then, it may be possible to allow for proof-of-principle and prevalidation studies to be conducted in accordance with the "intent" of GLP practices by requiring submitters to adequately document procedures and control measures and make experimental data open to regulatory review. "Best practices" for toxicogenomics can be established until formal procedures are adopted. This may be a more realistic solution that permits the advancement of science while addressing the need for QA and QC.

Validation as a Result of Procedural Changes

This third level of validation is necessary whenever a technical or methodologic change is introduced into the test. Such changes might, on one hand, be restricted to the microarray technology (e.g., modification or addition of sequences to a microarray, changes in data analysis procedures). Alternatively, they could involve the experimental design (e.g., dose, time, cell culture procedures). One consideration is that a distinction between minor and major procedural changes that might be incorporated into a test would help determine the extent of such validation necessary. Additionally, to facilitate the process, performance standards should be defined based upon the original validated test procedure. Minor changes would entail a demonstration of equivalence of results obtained with the modified test to that obtained from the validated test. Major changes would involve the need to define a new set of reference materials to be tested and a more extensive validation. Guidance on the use of performance standards and the elements comprising them have been published (ICCVAM 2003) and have been employed for *in vitro* dermal corrosion assessment methods (ICCVAM 2004). Such guidance can also help facilitate the establishment performance standards for toxicogenomics-based test methods in which procedural modifications have been introduced after an initial validation exercise, thereby providing a basis for the comparison of reliability and accuracy of the modified method relative to the validated and accepted reference test method.

The concept of performance standards was originally developed to evaluate the acceptability (accuracy and reliability) of proposed test methods that are based on similar scientific principles and that measure or predict the same biologic or toxic effect as an accepted (previously validated) test method. Because some regulatory authorities and international test guidelines programs (e.g., OECD) have restrictions regarding the use of proprietary test methods (methods that are copyrighted, trademarked, or patented), performance standards also allow for the development and validation of comparable nonproprietary methods based on performance standards derived from the corresponding proprietary antecedent method. Under these circumstances, performance standards allow the characteristics and functional attributes of a proprietary method or technique to be described and offer a procedure for evaluating the performance of methods claimed to be substantially similar. A method that meets the established performance standards is

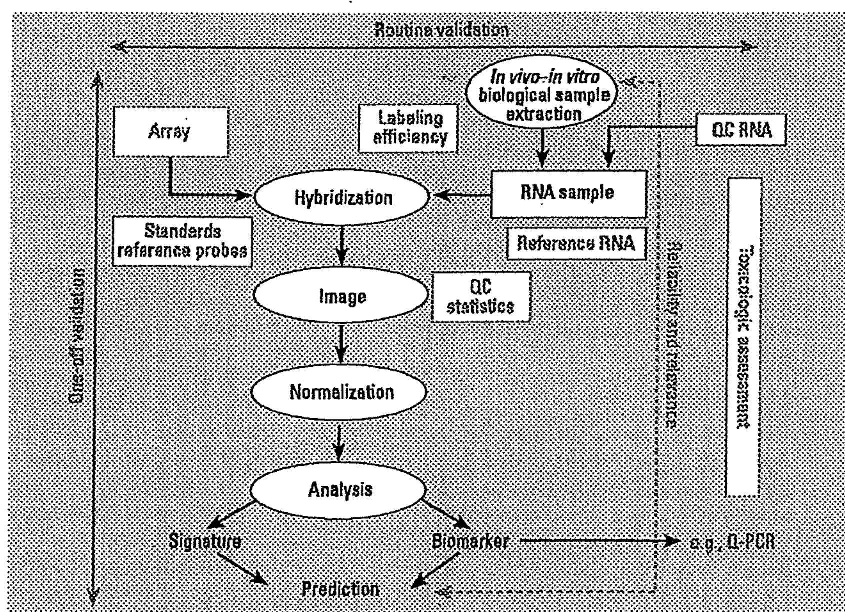

Figure 1. Scheme of the different steps in a toxicogenomics-based test. Three distinct levels were identified where validation is necessary: one-off validation (left), which should be performed once and is mainly related with the quality of the microarray and the instrumentation (blue); routine validation and QC (top), representing the ongoing requirements that are the responsibilities of the experimental toxicologist and the manufacturer (red); and the extent of validation necessary whenever a technical or methodologic change is introduced in the test (right): a method should meet the preestablished performance standards in order to be considered reliable and relevant as the original test method (green). Q-PCR, quantitative PCR.

considered sufficiently accurate and reliable for the specific testing purpose for which it is designed and is viewed as comparable with the original test method upon which it is based. If the correct performance standards have been developed, a method for which the results have the same accuracy and reliability as the original should by definition also be as relevant as the original method.

The conceptual framework and scope of performance standards could be expanded or adapted to include innovations or advancements in areas such as microarray or protein or metabolite separation and identification technology, where proposed improvements might or might not be generally or completely analogous to those in existing systems but would still enable similar applications. Performance standards could still provide a gauge for evaluating newer or revised technologies to ensure that their reliability and accuracy were at least comparable with that of existing acceptable techniques using similar chemicals even if essential test method components (i.e., structural, functional, and procedural elements of a validated test method to which a proposed, mechanistically and functionally similar test method should adhere) were not substantially similar.

This level of validation, which does not imply that a test needs to be completely revalidated, is of extreme importance for tests based on rapidly evolving technologies. It would be a mistake to immobilize these technologies by enforcement of a strict and inflexible validation approach that would hamper progress and test improvement. Finally, a periodic reassessment of a test method's performance (accuracy and reliability) employing established performance standards would help ensure adherence to essential test method components and the reliability and accuracy of the modified test method relative to the validated antecedent method (Hartung et al. 2004). Such assurance could be best established and reported by international validation bodies such as ECVAM and ICCVAM/NICEATM, which could track the history, performance, and validation status of a given test.

Data Management

The lack of robust QC procedures and capture of adequate metadata has caused problems with the analysis and reproducibility of array-based transcriptomics investigations. Consequently, the international MGED Society proposed standards for publication (Nature 2002) that were designed to clarify the MIAME guidelines (Brazma et al. 2001). As a result, a number of journals now require that articles containing microarray experiments must be compliant with the MIAME standard; some also require that the data integral to the article's conclusions be submitted to the ArrayExpress database at the EBI (European Bioinformatics Institute) (Brazma et al. 2003), GEO (Gene Expression Omnibus) at NCBI (National Center for Biotechnology Information) (Edgar et al. 2002), and CIBEX (Center for Information Biology Gene Expression database) at DDBJ (DNA Databank of Japan) (Ikeo et al. 2003)—the European, American, and Japanese database counterparts, respectively.

There is a critical need for public toxicogenomics databases because of the significant volume of data associated with these experiments, the complexity of comparing different gene annotations and splice variants across platforms, and the need for a resource for complex informatics analyses of the traditional toxicology and microarray data in parallel. However, to fully achieve the potential of this emerging interdisciplinary field, it is necessary that we move toward the establishment of a common public infrastructure for exchanging toxicogenomics data (Mattes et al. 2004). The infrastructure should address *a*) the technical problems involved in data upload, *b*) the demand for standardizing data models and exchange formats, *c*) the requirement for identifying minimal descriptors to represent the experiment, *d*) the necessity of defining parameters that assess and record data quality, and *e*) the challenge of creating standardized nomenclature and ontologies to describe biological data. The goal is also to create an internationally compatible informatics platform integrating toxicology/pathology data with transcriptomics, providing the scientific community with easy access to integrated data in a structured standard format, facilitating data analysis and data comparison, and enhancing the impact of the individual data sets and the comprehension of the molecular basis of actions of drugs or toxicants. Ultimately, such a knowledge-base could be maintained (respecting confidentiality as appropriate) as a reference for regulatory organizations to evaluate toxicogenomics and pharmacogenomics data submitted by registrants to those organizations.

The potential exists for the international development of this public infrastructure. As part of the collaborative undertaking with the International Life Sciences Institute Health and Environmental Sciences Institute (ILSI–HESI) Technical Committee on the Application of Genomics to Mechanism Based Risk Assessment (http://www.hesiglobal.org/committees), the European Molecular Biology Laboratory of the European Bioinformatics Institute (EMBL–EBI; Brazma et al. 2003; http://www.ebi.ac.uk/microarray/Projects/toxnutri/index.html), the National Institutes of Health/National Institutes of Health National Institute of Environmental Health Sciences National Center for Toxicogenomics (NCT; Waters et al. 2003; http://www.niehs.nih.gov/nct/), and the U.S. FDA NCT (Tong et al. 2003; http://www.fda.gov/nctr/science/centers/toxicoinformatics/index.htm) have worked closely together. The respective databases are based on the international standards developed by the MGED Society (Brazma et al. 2001; Spellman et al. 2002). After the very favorable response that the MIAME received from the microarray community and key scientific journals (Ball et al. 2002, 2004; Nature 2002), the MIAME checklist was extended to describe array-based toxicogenomics experiments. The MIAME-Tox checklist (MGED 2004) is an attempt to define the minimum information required to interpret unambiguously and potentially reproduce and verify array-based toxicogenomics experiments. MIAME-Tox also supports a number of other objectives, for example, linking data from different experimental domains within a study and linking several studies from one institution and exchanging toxicogenomics data sets among public databases. The major objective of MIAME-Tox is to guide development of toxicogenomics databases and data management software. Without a sufficient depth of data in these resources, the scientific community's opportunity to develop consensus on analysis and application of these data for risk assessment or screening may be limited. The availability of this level of information regarding platform specification, appropriate common reference standards, and the toxicologic study alone will facilitate the predictive value of toxicogenomics across different array-based platforms. This, in turn, will result in a greater appreciation of and confidence in the value of toxicogenomics within a regulatory context, such that testing strategies can be optimized, predictive alternative models can be identified, and animal use can be reduced (Supplemental Material, Section 2; http://ehp.niehs.nih.gov/members/2005/8247/suppl.pdf).

Moreover, the long-term provision of a MIAME-Tox–compliant database with a MAGE-ML (Microarray Gene Expression Markup Language) export is required for the long-term storage of toxicogenomics data. This would directly support the role of ECVAM, ICCVAM/NICEATM, and other validation bodies in the validation of toxicogenomics-based test methods.

The recommendations related to the technical and bioinformatics aspects of validation are listed in Table 2.

Regulatory Acceptance of Validated Toxicogenomics-Based Methods

Regulatory scientists are increasingly being called upon to consider incorporation of toxicogenomics data in regulatory assessment processes that involve evaluation of potential human health or environmental hazard and risk. Those scientists will need to be able to

judge the level of confidence to place in both *in vivo* and *in vitro* toxicogenomics-based test methods and the resulting data that might be submitted in support of regulatory decision making. Whether a method has been determined to be valid for a specific purpose will be an important factor for the consideration of its use for regulatory purposes. Furthermore, the level of confidence held by regulators will influence regulatory acceptance of methods and data, and will affect both the further pursuit of toxicogenomics technologies and technologic improvements and the extent of industry application of these technologies.

Potential uses of toxicogenomics data in the regulatory area. The potential of toxicogenomics-based methods in contributing to regulatory assessment processes is broad. Examples might include, but would not be limited to, obtaining microarray data from individual *in vivo* bioassays or *in vitro* cell or tissue-based assays or from batteries of assays, using conventional or high-throughput approaches. In accordance with the current developing state of the science, realistic possibilities for initial uses of toxicogenomics data in regulatory settings might be first in the realm of hazard assessment, such as to support chemical mechanism of action arguments. Other early uses might include aiding individual chemical/chemical mixture screening or ranking exercises to set priorities for toxicity testing or to sort chemicals into batches. These types of applications might involve identification of individual genes or gene patterns associated with particular toxic effects or pathways, adaptive responses, or metabolic pathways. However, global pattern recognition–type techniques are, as yet, not considered to be ready to fully replace traditional bioanalytical methods for predicting toxicity or elucidating information on mechanism of action or biochemical pathway component identification.

Using only human or animal *in vitro* or *in vivo* data derived from toxicogenomics technology to estimate such parameters as adverse/no adverse effect levels or to determine dose–response relationships for conducting risk assessments is regarded as a much longer term goal. However, for hazard assessment purposes, the possibility of considering toxicogenomics data along with other types of toxicologic information and data [e.g., from *in vivo* and *in vitro* studies, determinations of quantitative structure–activity relationships (QSAR) or SAR] in a weight-of-evidence approach on a case-by-case basis was not discounted. Regulatory bodies have begun to craft preliminary proposals, policies, and guidance for the submission and use of omics-type data in regulatory deliberations and to provide encouragement for the use and further development of the technology (U.S. EPA 2002; U.S. FDA 2005). Additionally, organizations such as the OECD are actively working with member countries on approaches that seek to harmonize the use of omics-derived information for hazard assessment related to health and environmental effects.

Harmonization of toxicogenomics-based test methods will first necessitate the standardization and validation of the specific test protocol(s) developed for a specific purpose(s), as conducted by international validation bodies such as ECVAM and ICCVAM/NICEATM. It will then be important for such organizations to interface with the OECD to ensure the appropriate crafting of harmonized OECD toxicogenomics-based test guidelines that are based upon standardized, adequately validated procedures, that are considered practical, and that permit consistent regulatory judgments.

Case for a modular approach to validation. Because of the extraordinary rate at which toxicogenomics technologies are evolving, current validation processes might need to adapt so as to accommodate the rapidly developing changes and advancements while still observing the basic tried-and-true validation principles. To meet this anticipated need, a modular approach to validation (Hartung et al. 2004) was considered, not to abridge the process but to allow for more flexibility in data collection and evaluation throughout the progressive changes that the technology will undergo. Typically, in the conventional validation procedures for an alternative test method, a sequential approach to the process is taken. The test protocol is first optimized and its transferability is determined. The resulting standardized method is then evaluated for within-lab and between-lab reproducibility and for its accuracy. Thus, an optimized, standardized protocol linked to specific test method elements and a prediction of outcome for given classes of chemicals are evaluated together for performance characteristics and applicability. Such a linear validation model, although effectively employed for other test methods, might not be optimal for dynamic test methods in which changes are rapidly introduced that improve or alter the protocol or the technology incorporated in the protocol in any substantive way. The linear validation model might result in unnecessary delays in incorporating innovations into toxicogenomics-type test methods. In contrast, with a modular approach to validation, which capitalizes on the fundamental classic concepts of validation as defined by ECVAM and ICCVAM (Balls et al. 1995; ICCVAM 1997, 2003), the different steps in the validation process are subdivided into independent modules, each of which can be assessed individually so that those components that have been completed need not undergo repeated validation. Further validation activities would instead be directed to only that part of the process flow where needed. The proposed model would accommodate validation of innovation affecting only a particular part of the sequence such that incorporation of advancements in a particular sector into testing strategies would less likely be impeded. At the same time, a modular approach to validation could efficiently handle information/data gaps that could be filled over time without derailing the validation stages already achieved. The modular approach, complemented with the use of performance standards (see "Validation as a Result of Procedural Changes" above), is expected to facilitate and help expedite the validation of the toxicogenomics technology and test methods that are based on toxicogenomics.

The modular approach follows the fundamental classic concepts of validation as defined by ECVAM and ICCVAM. Validation is defined as the process by which the relevance and reliability of a test method for a specific purpose are determined (Balls et al. 1995; ICCVAM 1997, 2003). Adequate validation

Table 2. Recommendations: focus on technology.

- Validation and QA/QC should be mandatory during the manufacturing of the arrays
- The array should undergo sequence verification and sequences should be available in the public domain
- MIAME guidelines should be adhered to
- Initially, develop "best practices" for toxicogenomics, including the interpretation of data and how to manage uncertainties and limitations
- Subsequently develop guidance for and adherence to GLPs for toxicogenomics experiments
- Common reference standards should be considered
- A workshop should be convened to address the development of standards for RNA sample preparation (and other biologic aspects of microarray analyses)
- Develop a "common" RNA standard including developing consensus about sources and maintenance of baseline data for regulatory and research purposes
- Studies should be MIAME-Tox compliant
- Performance standards should be developed and implemented to evaluate reliability and accuracy of test methods incorporating procedural modifications
- An ongoing dialogue should be maintained between scientists in the various relevant disciplines, including bioinformaticians, through meetings, published papers, and advisory/discussion panels (e.g., ILSI-HESI committee, NCT consortium, OECD panel)
- Ensure that validation efforts and QA/QC criteria are not restrictive to the technology or its advancement
- Explore whether toxicogenomics measurements can define toxicologic effects quantitatively
- Develop prediction models (e.g., algorithms) for toxicogenomics-based test methods
- Develop a data infrastructure for capturing, storing, and reporting toxicogenomics data
- Ensure continuation of financial support for long-term public database maintenance

involves development of a standardized test method protocol and assessment of the protocol's within- and between-laboratory variability, predictive capacity/accuracy, usefulness and limitations, and adherence to performance standards.

Standards for comparison. As technologic advancements are made and new, modified, or revised toxicogenomics-type test methods are put forward for consideration, it will be necessary to have a means by which the performance of proposed methodologies can be compared with that of existing (traditional and nontraditional) methods, especially those that employ animals. The lack of an approach rooted firmly in high-quality science could jeopardize attempts to seek or gain regulatory acceptance of toxicogenomics-based test methods and strategies. Evaluations of test method performance might be based on comparisons made between particular parameters, as dictated by the specific intent for which the assay was developed. Examples include the following:

- *In vivo–in vivo* study comparisons to examine concordance of gene changes with such factors as onset, duration, severity, dose, age, possible temporal changes of effects, and species differences
- *In vitro–in vivo* study comparisons to explore gene changes associated with a critical event or end point in an *in vitro* cell-based assay and an established *in vivo* biomarker of toxicity
- *In vitro–in vitro* study comparisons to analyze the responses of human and animal cell systems to xenobiotics
- Technologic comparisons to evaluate the effects of proposed technical improvements (e.g., comparing gene changes using different techniques of array/platform preparation)

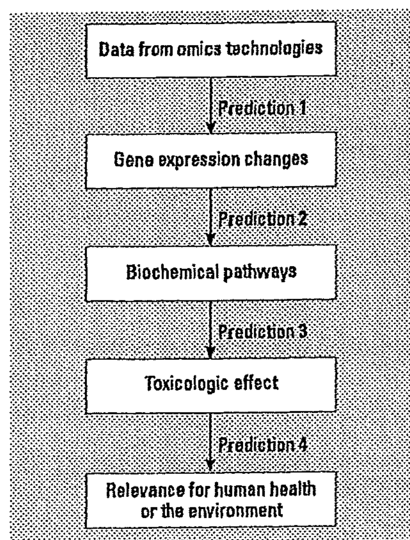

Figure 2. Process flow showing different independent prediction levels considered important in assessing validity of a toxicogenomics-based test method.

Accordingly, to detemine the appropriate types of validation activity and comparison in a given situation, it is important that the specific purpose of the proposed methodology and a detailed description of all relevant procedures be clearly elaborated (Balls et al. 1995; Hartung et al. 2004; ICCVAM 1997, 2003).

Toxicogenomics data from in vitro *systems and data relevance.* At the present time, toxicogenomics data derived from *in vitro* systems have been considered to have limited utility in regulatory applications. However, a great deal of interest exists for the further development of *in vitro*–based toxicogenomics methods, for an examination of their potential applicability in the regulatory arena, and for an appraisal of their potential for contributing to improvements in animal welfare. It is anticipated that technologic advancements will ultimately facilitate the use of *in vitro*–based methods as adjuncts to or surrogates for *in vivo*–based methods. Possible areas where validated *in vitro*–based toxicogenomics test methods might play a future role include *a*) preliminary assessments (prescreens), *b*) complementary testing that might assist in obtaining additional (e.g., mechanistic) information, and *c*) surrogate tests that could help in the refinement, reduction, and replacement of animals used for omics-based or traditional testing methods. One exciting aspect of toxicogenomics technology is the prospect of being able to identify species differences and/or similarities in the response to a xenobiotic. Although this is not viewed as near-term prospect, it obviously has potential applications for hazard and risk assessment purposes and could also have an impact on previous regulatory decisions when the technology becomes sufficiently advanced to permit such uses for it.

Additional regulatory acceptance issues. In considering approaches to validation, achieving regulatory acceptance of toxicogenomics-based methods or acceptance of information/data derived from such methods is an important goal. Regulators will be asked to evaluate whether data submitted using omics technologies can be used in support of a particular or broader based toxicologic, pharmacologic, or physiologic premise. For example, experiments using microarrays demonstrated increased expression of a cluster of related genes that was associated with enhanced activity and production of a microsomal enzyme important in the metabolic activation of a chemical to a toxic entity, which in turn was associated with a histopathologic biomarker lesion in the liver with a known human cancer correlate. Each of the events in this example can be thought of as a sequence of separate critical steps or information levels (Figure 2) that progressively connect omics data (from microarrays) to gene expression changes (increased expression), to a biochemical pathway (liver enzyme induction leading to toxic metabolite formation), to a toxicologic effect *in vivo* (liver lesion) with human relevance (cancer). Moving between two levels involves a prediction of outcome linking both steps. At each of these prediction junctures, regulators would be looking for evidence to scientifically substantiate moving to the next step and whether the prediction linking the levels (e.g., in this example, prediction 1, 2, 3, or 4 in Figure 2) was adequately validated. Theoretically, with this type of system, validated links could be established between any two levels. Technologic advancements or new information could be independently incorporated into a given level and considered and evaluated for the specific relevant prediction juncture. In this way, each of the prediction levels can be assessed independently and the validity of the links determined.

In the future toxicogenomics-based test methods may be shown to have been adequately validated and technically suitable for certain specific purposes, but regulatory acceptability and implementation will depend partly on whether the methods validated can be used for a given regulatory agency or program, that is, they are applicable to the products that fall within their regulatory purview. Some regulatory bodies may have internal peer-review processes, specific regulatory mandates, and/or regulatory assessment procedures that also have a role in the determination of test method applicability in regulatory programs, even though a test method may have been appropriately validated.

The widespread use of omics technologies will also bring about increasing demands on the regulatory community in terms of training of regulatory personnel in areas such as potential applications; data QC, analysis, and interpretation; statistical analysis; limitations of the technology; and how the information might be incorporated into safety, hazard, and risk assessment processes. To satisfy these needs, regulatory agencies have been engaging in developing and implementing training procedures, hiring scientists with the necessary technical knowledge and experience, establishing centers of excellence and dedicated laboratories focused specifically on the various omics and related informatics areas [e.g., National Center for Toxicological Research (U.S. FDA), NCT (NIEHS), Minister of Health Labour and Welfare–National Institute of Health Sciences Project in Japan, Netherlands Genomics Initiative, and EMBL–EBI, where informatic scientists are working with experimental practicioners and the MGED Society to ensure that transcriptomic experiments can be mapped on to regulatory toxicology studies]. In addition the regulatory arena has found that maintenance of open lines of communication with appropriate external scientists facilitates cooperation and the sharing of technical aspects, skills,

and practical experiences that help to broaden the collective knowledge base. Regardless, as the technology evolves further and finds wider application and acceptance, it will be necessary to address such fundamental matters as *a*) the generation, management, and interpretation of massive amounts of data; *b*) the consequent complex questions that will undoubtedly arise (e.g., what constitutes an adverse effect as identified using the technology; how does a given gene pattern correlate with a particular toxic end point or relate to onset, duration, and severity of effects, and to age, dose, and species?); and *c*) the limitations to the technology. Addressing such issues efficiently will warrant an ongoing dialogue between regulators and practitioners and a willingness to share relevant experiential and theoretical knowledge. Standard submission and presentation formats compatible with electronic data submission likely would need to be developed. Programs and staff would need to learn how information from the new technologies might be incorporated in regulatory practices and decision-making processes and would also have to face possible incongruities between toxicogenomics-derived data and existing or future submissions of conventional toxicity data. A number of regulatory authorities have already begun to contemplate and make provisions for this enormous and challenging task, but others may not yet have committed the resources to do so.

The recommendations related to regulatory acceptance and use of toxicogenomics-based test methods are listed in Table 3.

Conclusions

This workshop was organized as a result of the rapid growth and technologic advancements in the field of toxicogenomics; the promise it offers for numerous scientific arenas, especially human health and the environment; and the interest demonstrated by regulatory agencies as well as by the industrial sector. Consequently, it has become apparent that a considerable effort needs to be invested in the appropriate validation of both the technology alone and those test methods that incorporate the technology. The workshop provided a platform for technical experts in the field to become cognizant of the validation principles and regulatory issues to be encountered and for regulators and principal validation bodies to gain a better sense of those technologic aspects that would lend themselves to standardization, harmonization, and validation. Thus, this workshop was an important initiative that fostered an exchange of information fundamental to the ultimate adoption of toxicogenomics-based test methods for regulatory decision-making purposes. It is envisioned that the conclusions and recommendations that resulted will be a basis for future validation considerations for test method applications of toxicogenomics technologies in the regulatory arena and evaluating their potential utility for hazard/safety/risk assessments.

Several aspects of the validation of toxicogenomics that were identified as needing further exploration to help facilitate regulatory acceptance of future toxicogenomics-based test methods are as follows:

- Conduct toxicogenomics-based tests and the associated conventional toxicologic tests in parallel to *a*) generate comparative data supportive of the use of the former in place of the latter or *b*) provide relevant mechanistic data to help define the biological relevance of such responses within a toxicologic context
- Determine and understand the range of biologic and technical variability between experiments and between laboratories and ways to bring about greater reproducibility
- In the short term, favor defined biomarkers that are independent from technology platforms, and therefore are easier to validate; in the longer term, focus on pathway analysis (i.e., system biology approach) rather than just on individual genes
- Harmonize reference materials, QC measures, and data standards and develop compatible databases and informatics platforms that are key components of any validation strategy for a toxicologic method; this can only be achieved by promoting partnerships and collaborations among ongoing initiatives in toxicogenomics, standardization, and validation
- Determine performance standards for toxicogenomics-based test methods that will serve as the yardsticks for comparable test methods that are based on similar operational properties
- Define further the modular validation scheme that would allow keeping up with methodologic improvements and innovations without having to repeat the entire validation process but would, however, integrate ECVAM and ICCVAM principles of validation and acceptance.

Table 3. Recommendations: focus on regulatory acceptance of toxicogenomics-based methods.

- Build on and/or learn from previous and ongoing efforts in toxicogenomics, standardization, validation, and harmonization efforts where possible (e.g., MIAME, ICCVAM, ECVAM, NCT, EMBL–EBI, ILSI–HESI, U.S. FDA, U.S. EPA, OECD)
- Fund pilot programs to test possible validation strategies and processes
- Identify training needs and assist in developing training vehicles and ways of presenting the state-of-the-science to regulators and the regulated community (including electronic means)
- Maintain transparency of validation processes
- Explore additions, amendments, and revisions to ICCVAM and ECVAM validation guidance that would accommodate new and rapidly changing technologies
- Implement the modular approach to validation to accommodate existing knowledge and future technical developments
- Establish performance standards for toxicogenomics-based test methods and have them accommodate rapid technologic advancements and procedural modifications
- Explore, develop, and support sector-spanning worldwide harmonization entities
- Create confidence among regulators by involving them early on in discussions and various scientific forums that would facilitate application of the technology for regulatory purposes
- Encourage industry and other parties to share data, in part, to support validation comparisons
- Promote high-quality science in supporting the use and development of the technology for regulatory purposes to further protection of human health and the environment
- Consider opportunities for synergy between QSAR, pharmacokinetic, and pharmacodynamic modeling, and other *in silico* efforts and the toxicogenomics communities

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The expression of Hedgehog genes (*Ihh*, *Dhh*) and Hedgehog target genes (*Ptc1*, *Gli1*, *Coup-TfII*) is affected by estrogenic stimuli in the uterus of immature female rats

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Abstract

The objective of this study was to investigate the effects of estrogen receptor (ER) agonists and an ER antagonist on the expression of Hedgehog genes (Indian hedgehog: *Ihh*; Desert hedgehog: *Dhh*) and Hedgehog target genes (Patched 1: *Ptc1*; glioma-associated oncogene homolog 1: *Gli1*; chicken ovalbumin upstream promoter transcription factor II: *Coup-TfII*) in the rat uterus. Immature female rats were administered once with 17α-ethynyl estradiol (EE, an ER agonist), propyl pyrazole triole (PPT, an ERα-selective agonist), diarylpropionitrile (DPN, an ERβ-selective agonist), or ICI 182,780 (an ER antagonist). Expression of mRNA for *Ihh*, *Dhh*, and *Ptc1* was dose-dependently downregulated by EE in the uterus of immature rats, mediated by ER as confirmed by coadministration of ICI 182,780. The mRNA expression levels of *Ptc1*, *Gli1*, and *Coup-TfII* were simultaneously downregulated during the period in which the mRNA expression levels of *Ihh* and *Dhh* were downregulated in the uterus after administration of EE. PPT downregulated the transcription of *Ihh*, *Dhh*, *Ptc1*, *Gli1*, and *Coup-TfII*, indicating that expression of these genes was regulated by the ERα-dependent pathway. DPN also downregulated the transcription of *Ihh* and *Dhh*, although the effect was weaker than that of PPT, indicating that the regulation of uterine *Ihh* and *Dhh* transcription was also affected by the ERβ-dependent pathway. These results suggest that the expression of Hedgehog genes (*Ihh*, *Dhh*) and Hedgehog target genes (*Ptc1*, *Gli1*, *Coup-TfII*) is affected by estrogenic stimuli in the uterus of immature female rats.
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Introduction

Estrogen is a steroid hormone that plays an important role in the regulation of mammalian reproduction by regulating the transcription of specific genes. The transcriptional regulation of estrogen is mediated by two types of specific nuclear receptors, estrogen receptor alpha (ERα) (Green et al., 1986) and estrogen receptor beta (ERβ) (Kuiper et al., 1996; Mosselman et al., 1996). The uterus contains both ERα and ERβ, however, ERα is the predominant receptor (Couse et al., 1997; Rosenfeld et al., 2001). The study of *ERβ* knockout mice suggests that ERβ plays a role in modulation of the effects of ERα in the uterus. For example, in both the wild-type and ERβ knockout female mice, 17β-estradiol (E2) induced the expression of progesterone receptor (PR) in the stroma and glandular epithelial cells of the uterus. However, in the luminal epithelium, E2 inhibited the expression of PR in wild-type mice but had no effect on PR expression in these cells in ERβ knockout female mice, indicating that induction of PR is an ERα-mediated event and inhibition of epithelial PR is ERβ mediated (Weihua et al., 2000). ERα and ERβ regulate the expression of specific target genes by functioning as ligand-induced transcriptional factors to exert various physiological functions (Tasset et al., 1990;

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Mangelsdorf et al., 1995). Therefore, it is considered that the characterizations of the gene expression change by estrogenic stimuli provide important clues for understanding the influence of the estrogenic compounds.

Hedgehog proteins are involved in the regulation of cellular events at various stages of development from embryonic to adult in humans and many animal species (Ingham, 1998; Walterhouse et al., 1999; Ingham and McMahon, 2001; Walterhouse et al., 2003). *Drosophila* has a single Hedgehog gene, but higher vertebrates have three Hedgehog genes: Sonic hedgehog (*Shh*); Indian hedgehog (*Ihh*); and Desert hedgehog (*Dhh*) (Pathi et al., 2001). Regulation of diverse processes by Hedgehog proteins requires activation or suppression of the intracellular Hedgehog signaling pathway and the Hedgehog target genes, which finally leads to cell proliferation, growth arrest, differentiation, programmed cell death, or cell migration (Ingham, 1998; Ingham and McMahon, 2001; Oldak et al., 2001).

The Hedgehog signaling pathway is tightly regulated, and the signal is transmitted by two receptors, the twelve-transmembrane protein Patched (PTC) and the seven-transmembrane protein Smoothened (SMO). In the absence of Hedgehog proteins, PTC acts as a negative regulator of SMO. When present, Hedgehog proteins bind to PTC, and SMO is released from the inhibitory effect of PTC, then transcription factor GLI is translocated into the nucleus, where it regulates the transcription of Hedgehog target genes (Ingham and McMahon, 2001; Cohen, 2003). Dysregulation of the Hedgehog signaling pathway during development has been associated with significant birth defects, including basal cell nevus syndrome (Johnson et al., 1996) and holoprosencephaly (Belloni et al., 1996). In addition, dysregulation of the Hedgehog signaling pathway during postnatal life has been associated with cancers, including basal cell carcinoma (Xie et al., 1998) and sarcomas (Stein et al., 1999).

In the mammalian Hedgehog family, *Ihh* gene was reported as a progesterone-upregulated gene in the mouse uterus (Matsumoto et al., 2002; Takamoto et al., 2002). Estrogen and progesterone are steroid hormones that play a pivotal role in the regulation of female reproduction (DeMayo et al., 2002), and there is considerable biological evidence for cross-talk between the ER and PR signaling pathways (Kraus et al., 1995; Katzenellenbogen, 2000). For example, liganded PR-A and PR-B can each suppress E2-stimulated ER activity, with the magnitude of repression dependent on the PR isoform, ligand type (agonist or antagonist), PR levels, and ligand concentration (Kraus et al., 1995; Katzenellenbogen, 2000). Therefore, we hypothesized that the expression of Hedgehog-related genes may also be affected by estrogenic stimuli. However, only limited information is available whether estrogenic compounds influence the regulation of such gene expression.

Immature female rats are frequently used to investigate the regulatory actions of sex steroid hormones on various functions in the uterus. In the rat, the concentration of endogenous estrogen is consistently low throughout prepubertal development and starts to increase after 28 days of age. However, exposure to estrogenic compounds during the prepubertal period can induce an uterotrophic response (Noda et al., 2002; Naciff et al., 2003). This uterotrophic response in immature rats is being used as one of the standard assays to estimate estrogenic activity of different compounds *in vivo* (Owens and Ashby, 2002; Yamasaki et al., 2002; Kanno et al., 2003). Furthermore, it is considered that the immature female rats are suitable animal model to examine the influence of test compounds on the uterine gene expression via the ER (Naciff et al., 2003; Naciff et al., 2004; Lee et al., 2005; Katayama et al., 2006). Therefore, we used sexually immature female rats corresponding to a stage of high sensitivity to exogenous estrogenic stimulation to investigate the effects of ER agonists and an ER antagonist on the expression of Hedgehog genes and Hedgehog target genes in the uterus. Furthermore, we used four types of ER ligands: 17α-ethynyl estradiol (EE, an ER agonist used medically in oral contraceptives); propyl pyrazole triole (PPT, an ERα-selective agonist); diarylpropionitrile (DPN, an ERβ-selective agonist); and ICI 182,780 (ICI, an ER antagonist). PPT is a potent ERα agonist that has 410-fold higher binding affinity for ERα than ERβ and has been demonstrated to have almost no binding affinity to ERβ (Kraichely et al., 2000; Stauffer et al., 2000). In contrast, DPN is a potency-selective agonist for ERβ with a more than 70-fold higher binding affinity for ERβ than ERα (Meyers et al., 2001). We report here that ER agonists downregulate transcription of Hedgehog genes (*Ihh*, *Dhh*) and Hedgehog target genes (*Ptc1*, *Gli1*, *Coup-TfII*) in the uterus of immature female rats, predominantly via ERα-dependent pathway.

Materials and methods

Chemicals. 17α-Ethynyl estradiol (EE) and corn oil were obtained from Sigma-Aldrich Co. (St. Louis, MO, USA). Propyl pyrazole triole (PPT), diarylpropionitrile (DPN), and ICI 182,780 (ICI) were obtained from Tocris Cookson Inc. (Ellisville, MO, USA). Ethanol was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Corn oil containing 1% ethanol was prepared as the vehicle solution.

Animals. Thirteen-day-old, female Crj:CD (SD) IGS rats were obtained from Charles River Japan, Inc. (Kanagawa, Japan) with lactating maternal animals. After arrival, immature and maternal animals were acclimated for 5 days. Weaning and group assignment were performed on the day before administration (at 18 days old) to minimize the stress associated with weaning. One hundred animals in total were assigned to 20 groups (Experiment 1: 5 groups; Experiment 2: 12 groups; Experiment 3: 3 groups) to give similar intergroup variations in body weight among the resulting groups in each experiment. Animals were housed in polycarbonate cages (380×330×175 mm [width×depth×height], CLEA Japan, Inc., Tokyo, Japan). During the quarantine period, animals were accommodated in cages in groups of 10 immature animals and one maternal animal per cage. After group assignment, immature animals were accommodated at 5 animals per cage. The animal room was maintained at a temperature of 19.0 °C to 25.0 °C, with a relative humidity of 35.0% to 75.0%, and at a 12-h light/dark cycle. The animals were allowed free access to a pellet diet for experimental animals (MF, Oriental Yeast, Co., Ltd., Tokyo, Japan) and sterilized water. The animals were cared for in accordance with 'The Guidelines for Animal Experimentation' for our laboratory, Mitsubishi Chemical Safety Institute Ltd.

Study design. The study design was shown in Fig. 1. Rats were used for all experiments at 19 days of age. In Experiment 1 (dose-dependent response), animals were treated once by oral gavage with the vehicle or EE (0.3, 1, 3 μg/kg). In addition, to determine whether the gene expression changes caused by EE

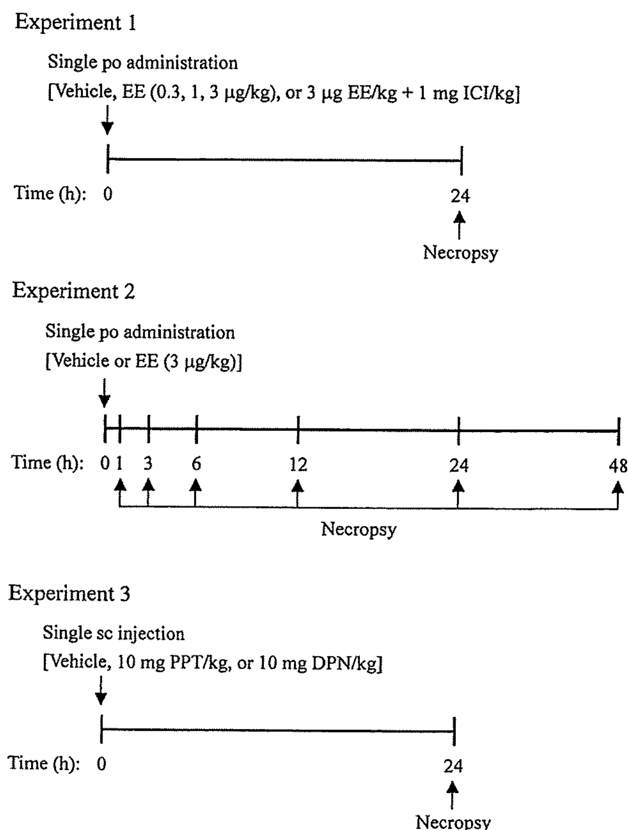

Fig. 1. Study design. Vehicle: corn oil containing 1% ethanol, EE: 17α-ethynyl estradiol (an ER agonist), ICI: ICI 182,780 (an ER antagonist), PPT: propyl pyrazole triole (an ERα-selective agonist), DPN: diarylpropionitrile (an ERβ-selective agonist).

were ER-mediated reactions, a sub-group was treated with ER antagonist (1 mg ICI/kg) and 3 μg EE/kg. In Experiment 2 (time-dependent response), animals were treated once by oral gavage with either the vehicle or 3 μg EE/kg. In Experiment 3, animals were treated once by subcutaneous injection with the vehicle, 10 mg PPT/kg, or 10 mg DPN/kg. The dose and administration route of EE and ICI were selected based on the results of preliminary experiment and published data (Kanno et al., 2001; Katayama et al., 2006). The dose and administration route of PPT and DPN were judged to be suitable to evaluate whether a certain phenomenon was predominantly regulated via either ERα or ERβ, based on the results of preliminary experiment and published data (Harris et al., 2002; Frasor et al., 2003; Tena-Sempere et al., 2004; Lee et al., 2005). Body weights were measured using an electronic balance (PM3000, Mettler Toledo K.K., Tokyo, Japan). The administration volume was 10 mL/kg, and was adjusted individually based on the body weight immediately before administration. Five animals were included in each treatment group. Animals were sacrificed by CO_2 asphyxiation in Experiments 1 and 3, at 24 h after administration; and in Experiment 2, at 1, 3, 6, 12, 24, and 48 h after administration. The uterus was removed from the body, placed on gauze, and cut at several sites to discharge gently the fluid in the uterus. The blotted uterine weight (weight of the uterus excluding the inner fluid) was measured using an electronic balance (Model AE260, Mettler Toledo K.K.). The uterus was submerged in the RNA preservative reagent RNA*later* (Ambion, Inc., Austin, TX, USA), kept at 4 °C overnight, and then was stored at −20 °C until processing for RNA isolation.

Isolation of total RNA. The uterus was homogenized in the dissolving and absorption liquid containing 1% 2-mercaptoethanol (TOYOBO CO., LTD., Osaka, Japan) for 300 s at −20 °C using an automatic sample preparation system (Twist Crusher HMX-2000, TOYOBO CO., LTD.). Total RNA was isolated using MagExtractor-RNA- (TOYOBO CO., LTD.) and an automatic nucleic acid extraction system (MagExtractor System MFX-2000, TOYOBO CO., LTD.) according to the manufacturer's recommended protocol, and was subsequently DNase-treated with RNase-free DNase I (TAKARA BIO INC., Shiga, Japan) for 30 min at 37 °C in the presence of RNase OUT (Invitrogen Corporation, Carlsbad, CA, USA). The amount of total RNA was determined using the RiboGreen RNA Quantitation kit (Molecular Probes, Inc., Eugene, OR, USA) or spectrophotometer (DU-7400, Beckman Coulter, Inc., Fullerton, CA, USA). The absence of genomic DNA contamination in the total RNA samples was confirmed by real-time PCR for each RNA sample without reverse transcriptase using TaqMan Rodent GAPDH Control Reagents VIC™(Applied Biosystems, Foster City, CA, USA), according to the manufacturer's recommended protocol.

Real-time reverse transcription-PCR. One-step real-time reverse transcription (RT)-PCR was performed to determine changes in the gene expression using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Total RNA (0.5 to 20 ng) isolated from each uterus was added to a reaction mixture containing forward primer, reverse primer, TaqMan probe, and TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems) in a final volume of 50 μL according to the manufacturer's instruction. Rat-specific primers and TaqMan probes were designed for the Hedgehog genes (*Ihh, Dhh*) and Hedgehog target genes (*Ptc1, Gli1, Coup-TfII*) using Primer Express software (Applied Biosystems) (Table 1). The mRNA expression of *Shh* was determined using primer and probe sets in the TaqMan Gene Expression Assays for rat *Shh* (Applied Biosystems). In this study, we selected 18S rRNA as a stable endogenous reference gene to normalize the target mRNA expression in the uterus (Katayama et al., 2006). TaqMan probes were labeled with a fluorescent reporter dye (FAM or VIC) at the 5′ end and a quencher dye (TAMRA) at the 3′ end. Thermal cycling conditions were as follows: 1 cycle of 30 min at 48 °C for reverse transcription; 1 cycle of 10 min at 95 °C for activation of DNA polymerase; 40 cycles of 15 s at 95 °C for denaturation and 1 min at 60 °C for annealing/extension. The expression levels of target gene and 18S rRNA in each sample were calculated based on the standard curve generated with the rat total RNA for the uterus (UNITECH. Co.,Ltd., Chiba, Japan) and ovary (Ambion, Inc.), or Rat Universal Reference Total RNA (BD Biosciences Clontech, Palo Alto, CA, USA). The expression level of target gene was then normalized by the expression level of 18S rRNA using TaqMan Ribosomal RNA Control Reagents (Applied Biosystems) to control the quantity of the isolated RNA. Real-time RT-PCR analyses were performed in duplicate on all five animals in each treatment group.

Statistical analysis. Data were expressed as the mean±S.D. for each group. Fold changes for uterine weights and gene expression data were expressed as the ratio of the mean value of the test compound group to the mean value of the vehicle group. Statistical analyses were performed using the SAS Proprietary Software Release 8.2 (SAS Institute, Inc., Cary, NC, USA). In experiment 1, statistical analysis among multiple groups was performed using Bartlett's test for homogeneity of variance. When the variance was homogenous, the data were analyzed by one-way analysis of variance test. When the differences were significant by one-way analysis of variance test, the data were analyzed by Williams' test. When the variance was not homogenous, the data were analyzed by Kruskal–Wallis test. When the differences were significant by Kruskal–Wallis test, the data were analyzed by the Shirley–Williams' test. In the all experiment, statistical analyses between 2 groups were performed using the *F*-test for homogeneity of variance (Experiment 1: 3 μg EE/kg vs 3 μg EE/kg +1 mg ICI/kg; Experiment 2: vehicle vs 3 μg EE/kg; Experiment 3: vehicle vs 10 mg PPT/kg, vehicle vs 10 mg DPN/kg). When the variance was homogeneous, the data were analyzed by Student's *t*-test. When the variance was not homogeneous, the data were analyzed by the Aspin–Welch's *t*-test. The *F*-test and Bartlett's test were conducted at the significance level of 5%. Williams' test and the Shirley–Williams' test were conducted at the one-tailed significance level of 5%. The other tests were conducted at the two-tailed significance level of 5%.

Results

In this study, *Shh* mRNA was not detected by the real-time RT-PCR analysis in the uterus of immature female rats,

Table 1
Sequences of primer and probe sets used for real-time RT-PCR analysis

Gene	Sequence	Accession no.	Amplicon size (bp)
Hedgehog gene			
Ihh			
Forward primer	5′-ACCCCACCTTCAGCGATGT-3′	XM_343590	78
Reverse primer	5′-GAGTCTCGATGACCTGGAAAGC-3′		
Probe	5′-CATTTTCCTGGACCGTGAGCCAAACAG-3′		
Dhh			
Forward primer	5′-CGTTACGTGCGCAAGCAA-3′	XM_343327	69
Reverse primer	5′-GGTCCGCTCGGGCATACT-3′		
Probe	5′-TGTGCCTCTGCTCTACAAGCAGTTTGTGC-3′		
Hedgehog target gene			
Ptc1			
Forward primer	5′-AGCGTACCTCCTAGGTAAGCCTC-3′	NM_053566	122
Reverse primer	5′-CGGCTTTATTCAGCATTTCCTC-3′		
Probe	5′-CGGTGGACAAACTTTGACCCCTTGG-3′		
Gli1[a]			
Forward primer	5′-TCTCCACAGTGGAGCCCAA-3′	XM_345832	76
Reverse primer	5′-CCCTCCGGCACAGTCAGT-3′		
Probe	5′-TGCTCTCTTCCCTGCCGGATCCT-3′		
Coup-TfII			
Forward primer	5′-CAATCAACTAGCCCTGAGCCA-3′	NM_080778	77
Reverse primer	5′-GCGCGCCGCCTTTT-3′		
Probe	5′-CCTCTCGCACCCTCGCACACAC-3′		

[a] Sequence of probe for *Gli1* was designed by the antisense strand.

regardless of the presence of EE, ICI, PPT, or DPN (data not shown). Therefore, *Ihh* and *Dhh* mRNAs were selected to evaluate the effects of estrogenic compounds on the expression of Hedgehog genes in immature rat uterus.

Experiment 1

To determine whether ER agonist (EE) and ER antagonist (ICI) influence the expression of Hedgehog genes (*Ihh*, *Dhh*) and Hedgehog target gene (*Ptc1*) in the uterus, immature female rats were treated once with the vehicle, EE (0.3, 1, 3 μg/kg), or EE (3 μg/kg) plus ICI (1 mg/kg).

The blotted uterine weights significantly increased in a dose-dependent manner by EE, in the dose range of 0.3–3 μg/kg, as compared with the vehicle group (Fig. 2A). The effect of EE (3 μg/kg) on the blotted uterine weight was significantly blocked by coadministration with ICI (1 mg/kg), indicating that the effect was ER-mediated (Fig. 2A).

The mRNA expression of *Ihh*, *Dhh*, and *Ptc1* significantly decreased in a dose-dependent manner by EE, in the dose range of 1–3 μg/kg, as compared with the vehicle group (Figs. 2B, C, D). The effects of EE (3 μg/kg) on the mRNA expression of *Ihh*, *Dhh*, and *Ptc1* were significantly blocked by coadministration with ICI (1 mg/kg), indicating that these effects were ER-mediated (Figs. 2B, C, D). However, coadministration with ICI was not able to fully block the inhibitory effect of EE on the mRNA expression of *Dhh* and *Ptc1*, while the effect on the mRNA expression of *Ihh* was completely abrogated by ICI. These results suggest that EE influences the mRNA expression of *Dhh* and *Ptc1* through the ER-dependent and ER-independent mechanisms.

Experiment 2

To determine the time-course changes in the expression of Hedgehog genes (*Ihh*, *Dhh*) and Hedgehog target genes (*Ptc1*, *Gli1*, *Coup-TfII*) in the uterus exposed to ER agonist, immature female rats were treated once with the vehicle or EE (3 μg/kg).

The blotted uterine weights significantly increased between 6 and 48 h after treatment with EE as compared with those in the time-matched vehicle groups, indicating that the treatment with EE at 3 μg/kg was enough to stimulate the immature rat uterus (Fig. 3A).

The mRNA expression of *Ihh* significantly decreased between 6 and 48 h after treatment with EE as compared with those in the time-matched vehicle groups (Fig. 3B). The decrease in the mRNA expression of *Ihh* in the EE group (3 μg/kg) reached maximum at 24 h (0.05-fold) after administration. In addition, the mRNA expression of *Ihh* fluctuated after treatment with the vehicle, indicating that it is important to set the time-matched vehicle group in time course experiment of gene expression profiling.

The mRNA expression of *Dhh* transiently and slightly increased 3 h (1.70-fold) after treatment with EE, but no statistical significance was noted in this change as compared with that in the time-matched vehicle group (Fig. 3C). Thereafter, the mRNA expression of *Dhh* decreased from 12 to 48 h after treatment with EE, as compared with those in the time-matched vehicle groups (Fig. 3C). A statistically significant difference was noted in the mRNA expression of *Dhh* 12 h (0.54-fold) and 48 h (0.39-fold) after treatment with EE as compared with those in the time-matched vehicle groups.

The mRNA expression of *Ptc1* significantly decreased between 3 and 48 h after treatment with EE as compared

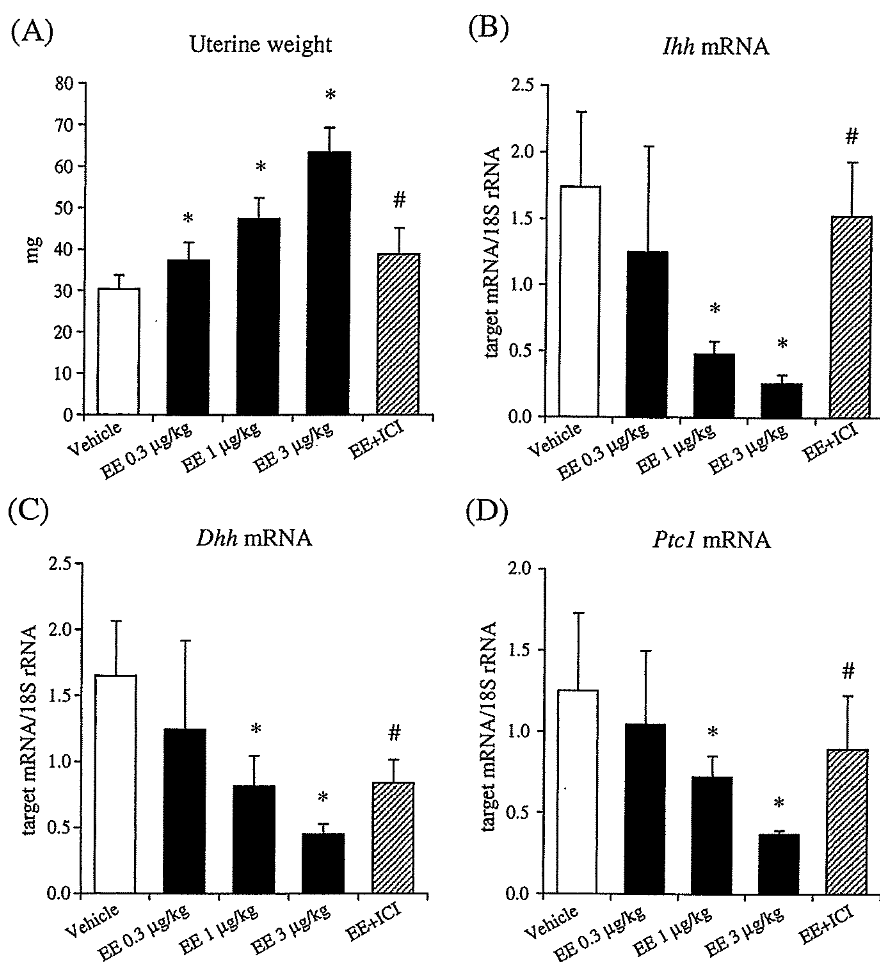

Fig. 2. Effects of EE and ICI on the uterine weights (A), the expression of Hedgehog genes (B, *Ihh*; C, *Dhh*), and Hedgehog target gene (D, *Ptc1*) of immature female rats. Each value represents the mean±S.D. of five animals per group. Vehicle: corn oil containing 1% ethanol, EE: 17α-ethynyl estradiol (an ER agonist), ICI: ICI 182,780 (an ER antagonist), EE+ICI: 3 μg EE/kg+1 mg ICI/kg. *: Significantly different from the vehicle group ($p<0.05$). #: Significantly different from the 3 μg EE/kg group ($p<0.05$).

with those in the time-matched vehicle groups (Fig. 3D). The decrease in the mRNA expression of *Ptc1* in the EE group (3 μg/kg) reached maximum at 24 h (0.31-fold) after administration.

The mRNA expression of *Gli1* transiently and significantly increased 3 h (3.72-fold) after treatment with EE as compared with that in the time-matched vehicle group (Fig. 3E). However, thereafter, the mRNA expression of *Gli1* significantly decreased more than those of the time-matched vehicle groups between 12 h (0.29-fold) and 48 h (0.23-fold) after administration (Fig. 3E).

The mRNA expression of *Coup-TfII* significantly decreased from 6 h after treatment with EE as compared with that in the time-matched vehicle group, and continued to decrease until 48 h after administration (Fig. 3F). Significant differences were noted between 6 h and 24 h (the lowest: 0.37-fold at 12 h) after administration.

Experiment 3

To determine whether a particular ER subtype is involved in the altered expression of Hedgehog genes (*Ihh*, *Dhh*) and Hedgehog target genes (*Ptc1*, *Gli1*, *Coup-TfII*) in the uterus exposed to ER agonists, immature female rats were treated once with the vehicle, PPT (10 mg/kg, ERα-selective agonist) or DPN (10 mg/kg, ERβ-selective agonist).

The blotted uterine weights significantly increased after treatment with either PPT or DPN as compared with the vehicle group (Fig. 4A). These results suggest that the increase of uterine weight by estrogenic compounds can occur through either ERα- or ERβ-dependent mechanisms. However, the magnitude of response caused by PPT (2.43-fold) was larger than that caused by DPN (1.32-fold).

The mRNA expression of *Ihh* and *Dhh* significantly decreased after treatment with either PPT or DPN as compared with the vehicle group (Figs. 4B, C). These results suggest that the downregulation of the mRNA expression of *Ihh* and *Dhh* by estrogenic compounds can occur through either ERα- or ERβ-dependent mechanism. However, the magnitude of response caused by PPT (*Ihh*: 0.08-fold; *Dhh*: 0.44-fold) was larger than those caused by DPN (*Ihh*: 0.60-fold; *Dhh*: 0.77-fold).

The mRNA expression of *Ptc1*, *Gli1*, and *Coup-TfII* significantly decreased after treatment with PPT (*Ptc1*: 0.26-fold; *Gli1*: 0.29-fold;

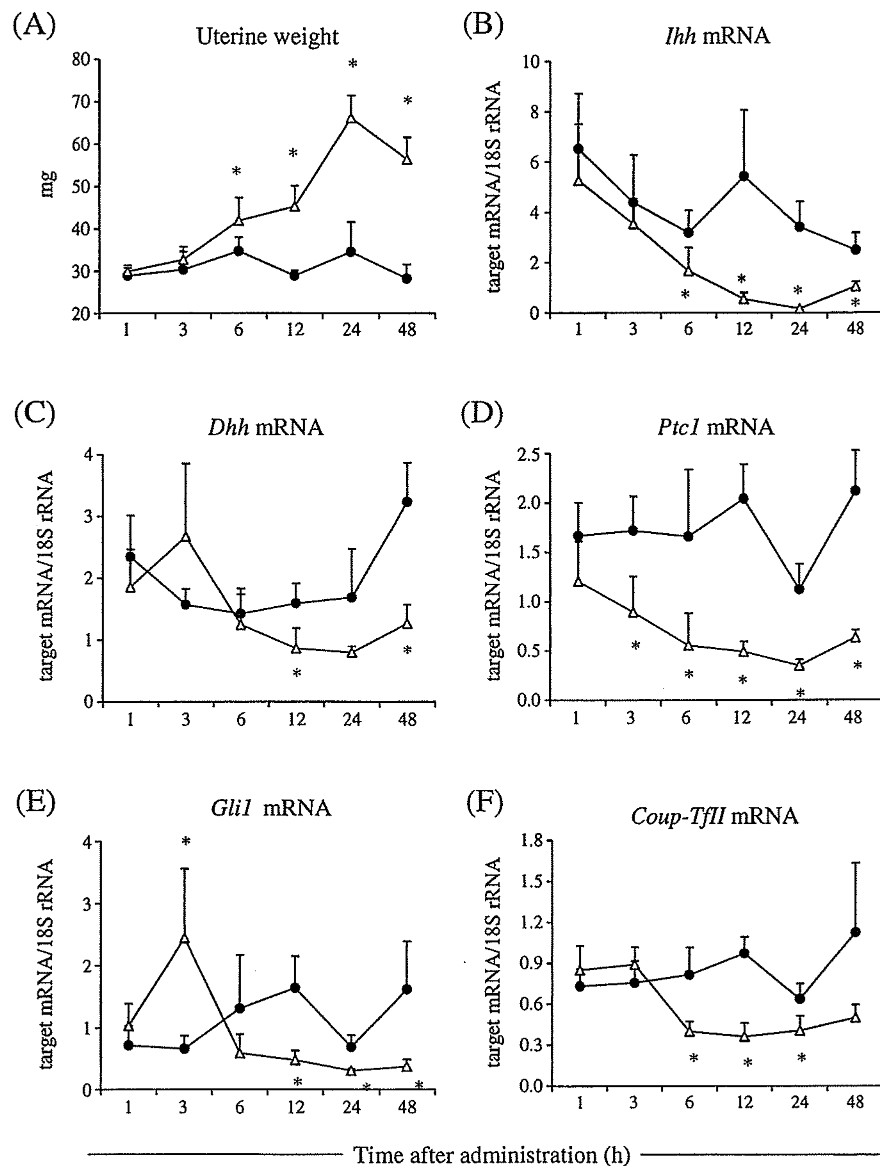

Fig. 3. Time-course changes in the uterine weights (A), the expression of Hedgehog genes (B, *Ihh*; C, *Dhh*), and Hedgehog target genes (D, *Ptc1*; E, *Gli1*; F, *Coup-TfII*) in the uterus of immature female rats exposed to EE. Each value represents the mean±S.D. of five animals per group. Vehicle: corn oil containing 1% ethanol, EE: 17α-ethynyl estradiol (an ER agonist). Closed circles indicate groups treated with the vehicle. Open triangles indicate groups treated with 3 μg EE/kg. *: Significantly different from the time-matched vehicle group ($p<0.05$).

Coup-TfII: 0.64-fold), but not with DPN, as compared with the vehicle group (Figs. 4D, E, F). These results suggest that the downregulation of the mRNA expression of *Ptc1*, *Gli1*, and *Coup-TfII* by estrogenic compounds can occur predominantly through the ERα-dependent mechanism.

Discussion

To understand the molecular events associated with the estrogenic activity of different chemicals, much information on gene expression changes after treatment with estrogenic compounds was reported in the rat uterus using microarray technology (Naciff et al., 2002; Naciff et al., 2003; Naciff et al., 2004). However, only limited information was available on whether estrogenic compounds influence the expression of Hedgehog-related genes. In this study, we demonstrated that estrogenic compounds affected the mRNA expression of Hedgehog genes and Hedgehog target genes in the uterus of immature rats.

In previous research, the *Ihh* gene was reported as a progesterone-upregulated gene (Matsumoto et al., 2002; Takamoto et al., 2002). Takamoto et al. (2002) demonstrated that the mRNA level of *Ihh* was induced within 3 h after a single injection of progesterone to ovariectomized mice, and that the induced *Ihh* mRNA and protein were localized to the luminal and glandular epithelial compartment of the endometrium. Matsumoto et al. (2002) used *in situ* hybridization to determine whether progesterone and estrogen differentially regulated the expression of *Ihh*. In ovariectomized mice, *Ihh* mRNA was expressed in uterine epithelial cells within 6 h after progesterone injection, and persisted until 24 h. On the other hand, estrogen was not effective

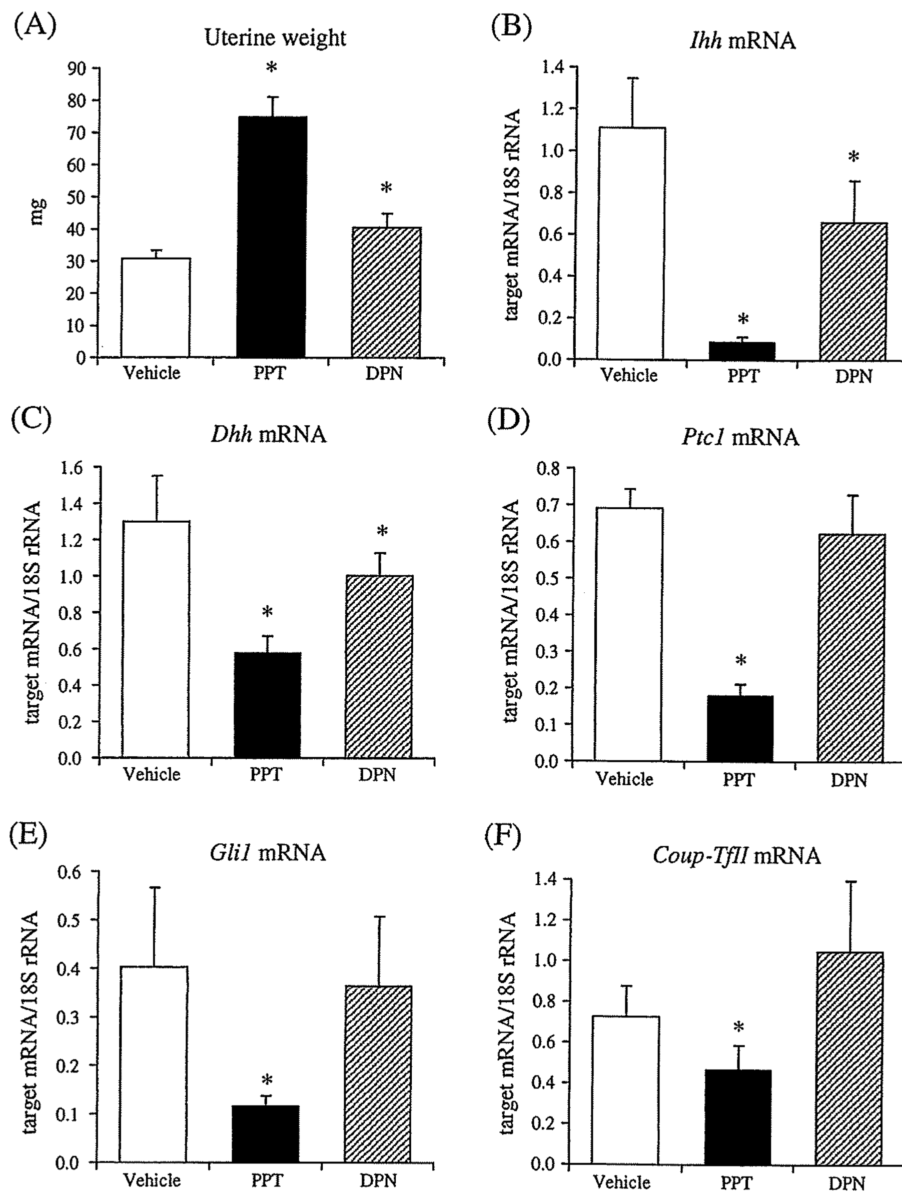

Fig. 4. Effects of PPT (10 mg/kg) and DPN (10 mg/kg) on the uterine weights (A), the expression of Hedgehog genes (B, *Ihh*; C, *Dhh*), and Hedgehog target genes (D, *Ptc1*; E, *Gli1*; F, *Coup-TfII*) in the uterus of immature female rats. Each value represents the mean±S.D. of five animals per group. Vehicle: corn oil containing 1% ethanol, PPT: propyl pyrazole triole (an ERα-selective agonist), DPN: diarylpropionitrile (an ERβ-selective agonist). *: Significantly different from the vehicle group ($p<0.05$).

in inducing this gene, and was not synergistic with it, but rather inhibited the progesterone-upregulated *Ihh* expression when the two hormones were given at the same time (Matsumoto et al., 2002). Recent work suggested that IHH signaling might play a role in preparation of the uterus for implantation during the peri-implantation period (Takamoto et al., 2002).

In this study, we demonstrated that the mRNA expression of *Ihh* and *Dhh* was dose-dependently downregulated by EE in the uterus of immature rats, and that these reactions were mediated by ER as confirmed by coadministration with ICI. PPT downregulated *Ihh* and *Dhh* mRNAs, indicating that uterine *Ihh* and *Dhh* mRNAs were tightly regulated by the ERα-dependent pathway. In addition, DPN also downregulated *Ihh* and *Dhh* mRNAs although these effects were weaker than those of PPT, indicating that the regulation of uterine *Ihh* and *Dhh* mRNAs were also affected by the ERβ-dependent pathway. In previous research, it was unclear whether the mRNA expression of *Ihh* and *Dhh* in the uterus was regulated by estrogenic compounds. However, the downregulation of *Ihh* and *Dhh* mRNAs in response to estrogenic stimuli was considered to be an important clue for understanding the uterine Hedgehog signaling pathway affected by ER agonists. To address this issue, we analyzed the promoter sequence using the 5′-flanking region of the rat *Ihh* and *Dhh* gene obtained from NCBI Rat Genome Resources (http://www.ncbi.nih.gov/genome/guide/rat/). These results indicated that the rat *Ihh* and *Dhh* promoter regions contained half-estrogen response element, half-cAMP response element, and GC box (data not shown). These ER-interacting

transcription factor-binding sequences are well known to involve the activation of gene transcription. However, in this study, the expression of *Ihh* and *Dhh* mRNAs in the uterus was downregulated by the ER agonist, especially the ERα-selective agonist. The GC box is the core sequence of binding site for specificity protein 1 (Sp1) and Kruppel-like factors (KLFs) family. Sp1 is the transcriptional activator that binds to GC-rich promoter elements. On the other hand, although KLFs bind to the promoter elements similar to Sp1, this family of proteins regulates the expression of various target genes by functioning as an activator of transcription, a repressor, or both (Cook et al., 1999; Lomberk and Urrutia, 2005). For example, it has been reported that KLF10 (also known Transforming growth factor-β Inducible Early Gene 1), a transcriptional repressor that binds to the GC box, was rapidly induced after treatment with 17β-estradiol in ER-positive human fetal osteoblastic cells (Tau et al., 1998). Therefore, we assumed that the ER signal activates the expression of some kinds of inhibitory transcription factor as the mechanism to downregulate the mRNA expression of *Ihh* and *Dhh* by the ERα-selective agonist (Zhang and Dufau, 2004; Lomberk and Urrutia, 2005). In this study, although the precise mechanisms of the ERα-selective agonist action on the mRNA expression of *Ihh* and *Dhh* remain to be elucidated, further study is needed to examine whether the transcriptional repressors such as KLFs are involved in the regulation of uterine *Ihh* and *Dhh* gene expression in response to estrogenic stimuli. In our study, the expression level of *ERβ* mRNA was about 100 times lower than that of *ERα* mRNA in the uterus of immature rats (Katayama et al., 2006). Therefore, a predominantly lower expression level of ERβ than ERα was considered as one of the reasons why the ERβ-selective agonist did not downregulate the mRNA expression level of *Ihh* and *Dhh* at the same level as the ERα-selective agonist.

If ER agonists interfere with Hedgehog signaling pathway through the alteration of the expression of Hedgehog ligands, then the mRNA expression of downstream target genes of Hedgehog should also be altered in the uterus of immature rats. In this study, we selected three Hedgehog target genes as follows: *Ptc1* (Ingham and McMahon, 2001; Yoon et al., 2002), *Gli1* (Lee et al., 1997; Ingham and McMahon, 2001; Dunaeva et al., 2003), and *Coup-TfII* (Krishnan et al., 1997a; Krishnan et al., 1997b). GLI1 (Sasaki et al., 1997; Teh et al., 2002; Yoon et al., 2002) and COUP-TFII (Pereira et al., 1999; Pereira et al., 2000; Zhang and Dufau, 2004; Takamoto et al., 2005) are transcription factors known to be involved in multiple signaling pathways by regulating the expression of various downstream target genes. In this study, the mRNA expression of *Ptc1* and *Coup-TfII* was downregulated on similar time courses to those of *Ihh* mRNA after EE administration. The time course of the mRNA expression of *Gli1* after EE administration was different from those of *Ptc1* and *Coup-TfII*, partially. The early phase change of *Gli1* mRNA was similar to that of *Dhh* mRNA, but not *Ihh* mRNA. DPN, a highly potent ERβ agonist, failed to downregulate the transcription of *Ptc1*, *Gli1*, and *Coup-TfII* mRNAs. However, PPT, an ERα agonist that poorly activates ERβ, downregulated the mRNA expression of *Ptc1*, *Gli1*, and *Coup-TfII*, indicating that these mRNA expressions were predominantly regulated by ERα-dependent pathway.

The Hedgehog signaling pathway is one of the key regulators of diverse cellular events. Therefore, it is necessary to precisely control the expression of Hedgehog gene through the various stages of development from embryonic to adult. Our findings in this study suggest that the expression of Hedgehog genes (*Ihh*, *Dhh*) and Hedgehog target genes (*Ptc1*, *Gli1*, *Coup-TfII*) is affected by estrogenic stimuli in the uterus of immature female rats. The experiment in immature female rats gives useful information to understand whether an ER ligand interferes with the expression of morphogenesis-related gene such as the Hedgehog gene. The obtained finding will provide the clues to predict the action of environmental estrogenic compounds *in vivo*.

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Differential Expression Patterns of *Wnt* and β-Catenin/TCF Target Genes in the Uterus of Immature Female Rats Exposed to 17α-Ethynyl Estradiol

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To characterize the effects of an estrogen receptor (ER) agonist on the gene expressions in the uterus, immature female rats were administered once orally with 17α-ethynyl estradiol (EE, 3 μg/kg), a potent ER agonist. We focused on four categories of sex steroid hormone receptor genes: well-known estrogen target genes, *Wnt* genes, and β-catenin/T-cell factor (TCF) target genes. *ERα*, *ERβ*, progesterone receptor, and androgen receptor mRNAs were all downregulated at 24 and/or 48 h after EE administration. Complement C3 and insulin-like growth factor 1 mRNAs were markedly induced after EE administration. Although the time courses of *Wnt4*, *Wnt5a*, and *Wnt7a* mRNA status varied until 12 h after EE administration, all of them were simultaneously downregulated at 24 and 48 h. The remarkable downregulation of *Wnt7a* mRNA in response to EE was considered to be important to understand the various uterine phenomena affected by ER agonists. In the β-catenin/TCF target genes, the downregulation of anti-Mullerian hormone type 2 receptor and bone morphogenetic protein 4 mRNA after EE administration appeared to be closely related to the downregulation of *Wnt7a*. The upregulation of cyclin D1 and follistatin mRNA at the early phase after EE administration was considered to have been affected by the upregulation of *Wnt4*. These results indicate that an ER agonist influences not only the mRNA expression of sex steroid hormone receptor genes and well-known estrogen target genes but also *Wnt* genes (*Wnt4*, *Wnt5a*, *Wnt7a*) and β-catenin/TCF target genes in the uterus of immature rats, indicating that their molecules are the potential players affected by estrogenic stimuli.

Key Words: estrogen receptor agonist; uterus; *Wnt* genes; β-catenin/TCF target genes.

The evidence that several synthetic compounds released into the environment may cause developmental and reproductive abnormalities in wildlife by disrupting normal endocrine functions has increased the concern about potential adverse human health effects from such endocrine disruptors (Colborn *et al.*, 1993; Kavlock *et al.*, 1996). Among the compounds considered as endocrine disruptors, many of them have estrogenic activity (Kanno *et al.*, 2001, 2003; Owens and Ashby, 2002). However, there are still many unresolved aspects about the molecular mechanism in the process to induce abnormal differentiation of the female reproductive tract exposed to estrogenic compounds.

Estrogen is a steroid hormone that plays a pivotal role in the regulation of mammalian reproduction and acts by regulating the transcription of specific genes through the specific nuclear receptors, estrogen receptor alpha (ERα) and estrogen receptor beta (ERβ) (DeMayo *et al.*, 2002). Therefore, changes in the expression of estrogen target genes are considered to be a useful index for evaluating the estrogenic activity of synthetic compounds. However, it is difficult to predict the full range of effects of estrogenic compounds from changes of the expression level of only the well-known estrogen target genes. Therefore, we considered whether there was any possible candidate with a further broad range of effects among the genes responding to estrogenic compounds.

Wnt genes encode a large family of secreted cysteine-rich proteins that play key roles as intercellular signaling molecules in embryonic development (Wodarz and Nusse, 1998). In the Wnt family, Wnt4, Wnt5a, and Wnt7a play important roles in the female reproductive system (Heikkila *et al.*, 2001). Wnt4 expression is crucial for the formation of Mullerian ducts, and thus both male and female *Wnt4*-deficient mice completely lack Mullerian ducts (Vainio *et al.*, 1999). *Wnt5a*-deficient mice die at birth due to a failure to complete anteroposterior body axis development (Yamaguchi *et al.*, 1999). Mericskay *et al.* (2004) demonstrated that Wnt5a is required to appropriately establish the development of the posterior region of the

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female reproductive tract. Furthermore, although the oviduct, uterine, and cervical compartments of the female reproductive tract developed in the absence of *Wnt5a*, the mutant uterus failed to form glands that are essential for adult function (Mericskay *et al.*, 2004). *Wnt7a* is expressed in the luminal epithelial cells of the fetal Mullerian tracts and is maintained at high levels in the adult uterine luminal epithelium (Miller *et al.*, 1998b). *Wnt7a*-deficient mice are viable, but *Wnt7a* mutant males are infertile due to the consequences of ectopic Mullerian ducts. Mutant females are sterile because of the abnormal development of the oviduct and uterus, both of which are Mullerian duct derivatives (Miller and Sassoon, 1998; Parr and McMahon, 1998). The abnormal female reproductive tract caused by the deficiency of *Wnt7a* closely resembles the abnormalities in female humans and mice prenatally exposed to diethylstilbestrol (Miller *et al.*, 1998a).

In the Wnt signaling pathway, at least three kinds of the Wnt/β-catenin pathway, the Wnt/c-Jun N-terminal kinase pathway (Wnt/JNK pathway), and the Wnt/Ca^{2+} pathway are identified (Kuhl *et al.*, 2000; Veeman *et al.*, 2003; Willert and Nusse, 1998). The Wnt/β-catenin pathway regulates the transcription of various target genes via stabilized β-catenin and T-cell factor/lymphoid enhancer factor (TCF/LEF) family members. If a certain estrogenic compound influences the expressions of *Wnt* genes and/or β-catenin/TCF target genes, then the compound may cause altered development of the female reproductive tract. However, only limited information is available whether estrogenic compounds influence the regulation of such gene expression.

The objective of this study was to investigate the effects of ER agonist on the expression of *Wnt* genes and β-catenin/TCF target genes. In this study, we used 17α-ethynyl estradiol (EE) as an ER agonist. EE is the estrogenic chemical used medically in oral contraceptive. It is estimated that each year about 3% of women in the United States and Europe who use oral contraceptives become pregnant accidentally, primarily because of missed pills (Thayer *et al.*, 2001; Timms *et al.*, 2005). Oral contraceptive pills may be taken for many months until the unplanned and unexpected pregnancy is discovered (Li *et al.*, 1995; Timms *et al.*, 2005). Therefore, there is concern about the effect of EE on the reproductive tract including uterus because it has the possibility of becoming an unexpected source of exposure of human fetuses to estrogenic compounds. We report here that EE influences the mRNA expression of *Wnt* genes (*Wnt4*, *Wnt5a*, *Wnt7a*) and β-catenin/TCF target genes in the uterus of immature rat.

MATERIALS AND METHODS

Chemicals. EE (purity: 99%) and corn oil were obtained from Sigma-Aldrich Co. (St. Louis, MO). Ethanol was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Corn oil containing 1% ethanol was prepared as the vehicle solution.

Animals. Thirteen-day-old, female Crj:CD(SD) IGS rats were obtained from Charles River Japan, Inc. (Kanagawa, Japan) with lactating maternal animals. After arrival, immature and maternal animals were acclimated for 5 days. Weaning and group assignment were performed on the day before administration (at 18 days old) to minimize the stress associated with weaning. Sixty animals were assigned to 12 experimental groups to give similar intergroup variations in body weight among the resulting groups. Animals were housed in polycarbonate cages (380 × 330 × 175 mm [width × depth × height], CLEA Japan, Inc., Tokyo, Japan). During the quarantine period, animals were accommodated in cages in groups of 10 immature animals and one maternal animal per cage. After group assignment, immature animals were accommodated at five animals per cage. The animal room was maintained at a temperature of 19.0–25.0°C, with a relative humidity of 35.0–75.0%, and at a 12 h light/dark cycle. The animals were allowed free access to a pellet diet for experimental animals (MF, Oriental Yeast, Co., Ltd., Tokyo, Japan) and sterilized water. The animals were cared for in accordance with "The Guidelines for Animal Experimentation" for our laboratory, Mitsubishi Chemical Safety Institute Ltd.

Study design. We used sexually immature female rats in which significant ovarian estrogen synthesis and regulation by the hypothalamic-pituitary-gonadal axis had not been initiated. Rats were used for the experiment at 19 days of age. Animals were administered once by oral gavage with either the vehicle or 3 μg/kg EE. The dose of EE was selected at a dose known to cause hypertrophy of the uterus in a 3-day uterotrophic assay (Kanno *et al.*, 2001). Initial body weight before the administration and final body weight before the necropsy were measured using an electronic balance (PM3000, Mettler Toledo K.K., Tokyo, Japan). The administration volume was 10 ml/kg and was adjusted individually based on the initial body weight before administration. Five animals were included in each treatment group. Animals were euthanized by CO_2 asphyxiation at 1, 3, 6, 12, 24, and 48 h after administration. The uterus was removed from the body, placed on gauze, and cut at several sites to discharge gently the fluid in the uterus. The absolute "blotted uterine weight" (weight of the uterus excluded the inner fluid) was measured using an electronic balance (Model AE260, Mettler Toledo K.K.). In addition, the relative weight of the uterus from each animal was calculated by dividing the absolute blotted uterine weight by final body weight before the necropsy. The uterus was submerged in the RNA preservative reagent RNAlater (Ambion, Inc., Austin, TX), kept at 4°C overnight, and then was stored at −20°C until processing for RNA isolation.

Isolation of total RNA. The uterus was homogenized in the dissolving and absorption liquid containing 1% 2-mercaptoethanol (TOYOBO Co., Ltd., Osaka, Japan) for 300 s at −20°C using an automatic sample preparation system (Twist Crusher HMX-2000, TOYOBO Co., Ltd.). Total RNA was isolated using MagExtractor-RNA- (TOYOBO Co., Ltd.) and an automatic nucleic acid extraction system (MagExtractor System MFX-2000, TOYOBO Co., Ltd.) according to the manufacturer's recommended protocol and was subsequently DNase treated with RNase-free DNase I (TAKARA BIO INC., Shiga, Japan) for 30 min at 37°C in the presence of RNase OUT (Invitrogen Corporation, Carlsbad, CA). The amount of total RNA was determined using the RiboGreen RNA Quantitation kit (Molecular Probes, Inc., Eugene, OR) or spectrophotometer (DU-7400, Beckman Coulter, Inc., Fullerton, CA). The absence of genomic DNA contamination in the total RNA samples was confirmed by real-time polymerase chain reaction (PCR) for each RNA sample without reverse transcriptase using TaqMan Rodent GAPDH Control Reagents VIC (Applied Biosystems, Foster City, CA), according to the manufacturer's recommended protocol.

Real-time reverse transcription–PCR. One-step real-time reverse transcription (RT)–PCR was performed to determine changes in gene expression using the ABI PRISM 7700 Sequence Detection System (Applied Biosystems). Total RNA (0.5–20 ng) isolated from each uterus was added to a reaction mixture containing forward primer, reverse primer, TaqMan probe, and TaqMan One-Step RT-PCR Master Mix Reagents (Applied Biosystems) in a final volume of 50 μl according to the manufacturer's instruction. Rat-specific

TABLE 1
Sequences of Primer and Probe Sets Used for Real-Time RT-PCR Analysis

Gene		Sequence (5′–3′)	Accession number	Amplicon size (bp)
ERα	Forward primer	ccaccgagtcctggacaaga	X61098	69
	Reverse primer	tgcagagtcaggccagctt		
	Probe	cacagacactttgatccacttgatggcc		
ERβ	Forward primer	gggtgattgcgaagagtggta	NM_012754	91
	Reverse primer	ctgatgtgcctgacgtgagaa		
	Probe	cgactggccaacctcctgatgcttc		
PR	Forward primer	caacacaaagcccgacacttc	NM_022847	108
	Reverse primer	cggaaacctggcagagactta		
	Probe	ttgctgaccagtctcaaccaactaggcg		
AR	Forward primer	atgaccagatggcagtcattcag	NM_012502	141
	Reverse primer	acttgtgcatgcgatactcattg		
	Probe	aggatgctctactttgcacctgacctggt		
Complement C3	Forward primer	agttctttaacgtgggacttatcca	NM_016994	138
	Reverse primer	acatttcattgtggcacagctt		
	Probe	atgcacccggttctatcatccggaga		
Igf1	Forward primer	gtgatctgaggaggctggagat	X06043	71
	Reverse primer	ggcccggatggaacga		
	Probe	actgtgctccgctgaagcctacaaagtc		
Wnt4	Forward primer	gaaccggcgctggaact	NM_053402	70
	Reverse primer	ggtcccttgtgtcaccacctt		
	Probe	ttccacactggactccctgcctgtc		
Wnt5a	Forward primer	gcgtggctatgaccagtttaaga	NM_022631	75
	Reverse primer	ttgacatagcagcaccagtgaaa		
	Probe	cagaccgaacgctgccattgcaa		
Wnt7a	Forward primer	gcccacctttctgaagatcaag	XM_342723	103
	Reverse primer	tgggtcctcttcacagtaattgg		
	Probe	ccgtaagcccatggacactgatctgg		
Amhr2	Forward primer	ggctctactactgtgggagatcct	NM_030998	90
	Reverse primer	tctgcctcataagccaattgaa		
	Probe	tgttccgatttgaggcctgaccaca		
Bmp4	Forward primer	tggacacctcatcacacgactac	NM_012827	111
	Reverse primer	tgcttttcccgggtccat		
	Probe	ttcgatgtgagccctgcagtccttc		
Fgf9[a]	Forward primer	acccaggtggtcacttagcaa	NM_012952	114
	Reverse primer	atggctcccttaggtgaagttg		
	Probe	cggtaccgcgtcctgcacacc		
Cyclin D1	Forward primer	cctctcctgctaccgcacaa	NM_171992	111
	Reverse primer	cgcaggcttgactccagaag		
	Probe	cggactgcctccgtgcctgc		
Follistatin	Forward primer	cggcgtactgcttgaagtga	NM_012561	100
	Reverse primer	agggaagctgtagtcctggtctt		
	Probe	tccggatcttgcaactccatctcgg		
Fibronectin	Forward primer	tgcgctccattccaccttat	NM_019143	110
	Reverse primer	tccctggcttggtcgtacac		
	Probe	cgctccaaggattggcttcaagctg		
Mmp7	Forward primer	ctctaggccatgcctttgca	NM_012864	140
	Reverse primer	acccagagagtggccaagttc		
	Probe	tcaggagtgaacttcctgtttgttgcca		

[a]Sequences of primer and probe sets for *Fgf9* were designed by the antisense strand.

primers and TaqMan probes were designed for the genes of interest (Table 1) using Primer Express software (Applied Biosystems). The mRNA expression of glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) was determined using primer and probe sets in the TaqMan Rodent GAPDH Control Reagents VIC (Applied Biosystems). TaqMan probes were labeled with a fluorescent reporter dye (FAM or VIC) at the 5′ end and a quencher dye (TAMRA) at the 3′ end. Thermal cycling conditions were as follows: 1 cycle of 30 min at 48°C for reverse transcription, 1 cycle of 10 min at 95°C for activation of DNA polymerase, 40 cycles of 15 s at 95°C for denaturation, and 1 min at 60°C for annealing/extension. The expression levels of target gene and 18S rRNA in each sample were calculated based on the standard curve generated with the rat total RNA for the uterus (UNITECH Co., Ltd., Chiba, Japan) or ovary (Ambion, Inc.). The expression level of target gene was then normalized by the expression level of 18S rRNA using TaqMan Ribosomal RNA Control

Reagents (Applied Biosystems) to control the quantity of the isolated RNA. Real-time RT-PCR analyses were performed in duplicate on all five animals in each treatment group.

Statistical analysis. The data of each group were expressed as the means ± SDs. Fold changes for uterine weights and gene expression data were expressed as the ratio of the mean value of the group treated with EE (3 μg/kg) to the mean value of the time-matched vehicle group. Differences of body weights, uterine weights, and gene expression data in the EE treatment group from those in the time-matched vehicle group were analyzed for statistical significance. *F*-test was applied to analyze the homogeneity of the variance. When the variance was homogeneous, Student's *t*-test was performed. When the variance was not homogeneous, Aspin-Welch' *t*-test was performed. The statistical analysis was performed with SAS Proprietary Software Release 8.2 (SAS Institute, Inc., Cary, NC). Significance was determined by a two-tailed significance level of 5%.

RESULTS

Effects of EE on the Body Weight in Immature Female Rats

No statistically significant differences were noted in body weights between any of the 3-μg/kg EE groups and the time-matched vehicle groups (Table 2). Since there were no abnormal clinical signs observed in any groups, 3 μg/kg of EE was considered not to produce serious toxicity in the animals.

Effects of EE on the Uterine Weight in Immature Female Rats

Uterine weights significantly increased between 6 and 48 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups (Fig. 1, Table 3). An increase of the relative uterine weight in the 3-μg/kg EE group reached a peak at 24 h (1.99-fold) after administration.

Effects of EE on the Expression of Reference Genes in the Uterus of Immature Female Rats

Reference genes, which are often referred to as housekeeping genes, are frequently used to normalize mRNA levels between different samples. However, the expression level of these genes may vary in different tissues, different cell types, and different disease stages, so the selection of the reference genes is critical for the interpretation of the expression data. Therefore, to determine a stable endogenous reference gene in the uterus after treatment with ER agonist, time-course changes were measured in the expressions of commonly used reference genes, *Gapdh* mRNA and 18S rRNA. Firstly, the expression of *Gapdh* mRNA and 18S rRNA were normalized with an amount of total RNA. The expression of *Gapdh* mRNA normalized with total RNA significantly increased between 3 and 12 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups (Fig. 2B). However, no statistically significant differences were noted in the expression of 18S rRNA normalized with total RNA between any of the 3-μg/kg EE groups and the time-matched vehicle groups (Fig. 2A). Therefore, 18S rRNA was judged to be suitable as a stable endogenous reference gene

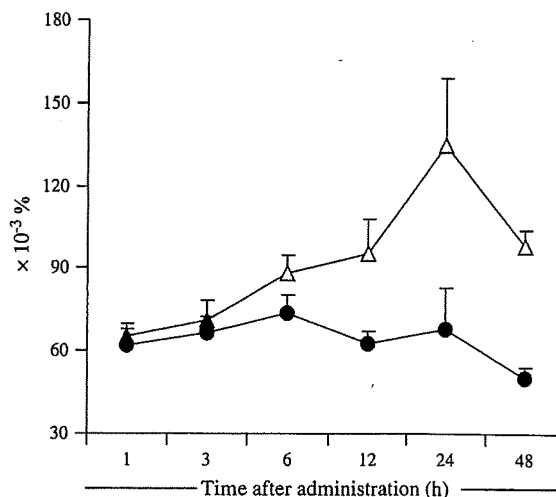

FIG. 1. Effects of EE on the uterine weight in immature female rats. Wet weight of uterus was normalized to final body weight (absolute blotted uterine weight/final body weight × 10^{-3}%). Each value represents the mean ± SD of five animals per group. Closed circles indicate groups treated with the vehicle. Triangles indicate groups treated with EE at 3 μg/kg. Open triangles indicate a statistical significance from the time-matched vehicle group ($p < 0.05$).

TABLE 2
Effects of EE on the Body Weight in Immature Female Rats

	Initial body weight before the administration[a]		Final body weight before the necropsy[b]	
Group	Vehicle	EE (3 μg/kg)	Vehicle	EE (3 μg/kg)
1 h[c]	43.9 ± 3.1	44.4 ± 2.1	46.9 ± 3.5	46.1 ± 3.3
3 h	43.9 ± 2.8	44.4 ± 2.1	45.4 ± 3.0	46.1 ± 3.0
6 h	44.1 ± 2.3	44.4 ± 2.2	47.0 ± 2.8	47.3 ± 3.5
12 h	44.3 ± 2.1	44.4 ± 2.3	46.1 ± 2.3	47.5 ± 2.2
24 h	44.4 ± 2.0	44.3 ± 2.3	51.1 ± 3.5	49.8 ± 4.7
48 h	44.4 ± 2.0	44.4 ± 2.2	56.0 ± 4.9	57.5 ± 3.1

Note. Each value represents the mean ± SD of five animals per group. No values were significantly different from those in the time-matched vehicle group.
[a]19 days old (referring to the date of their birth as day 0).
[b]19 days old (1-, 3-, 6-, and 12-h groups), 20 days old (24-h group), and 21 days old (48-h group).
[c]Time after administration.

TABLE 3
Time-Course Changes in Organ Weight and Gene Expression in the Uterus of Immature Female Rats Treated with EE

	Time after administration (h)											
Endpoint	1		3		6		12		24		48	
Uterine weight	1.05		1.07		1.19	+	1.52	+	1.99	+	1.95	+
Sex steroid hormone receptor gene												
ERα	1.24		1.23		0.99		0.80		0.63	−	0.57	−
ERβ	2.01	+	1.75		0.25	−	0.69		0.61	−	0.46	
PR	1.27		1.27		0.71		0.60	−	0.40	−	0.51	−
AR	2.16		1.93	+	1.01		0.92		0.96		0.50	−
Well-known estrogen target gene												
Complement C3	0.86		1.73		7.17	+	16.80	+	43.90	+	77.82	+
Igf1	1.95		9.73	+	3.68	+	3.90	+	1.77		0.52	−
Wnt gene												
Wnt4	0.83		2.39		1.71	+	1.95	+	0.51	−	0.43	−
Wnt5a	1.06		2.12	+	1.04		1.08		0.45	−	0.39	−
Wnt7a	0.70	−	0.70		0.56	−	0.48	−	0.15	−	0.28	−
β-catenin/TCF target gene												
Amhr2	1.08		0.91		0.14	−	0.23	−	0.18	−	0.25	
Bmp4	1.01		0.90		0.25	−	0.13	−	0.65	−	0.22	−
Fgf9	0.76		0.69		0.90		1.06		0.40	−	0.66	−
Cyclin D1	1.32		1.89	+	1.87	+	1.67	+	0.45	−	0.79	
Follistatin	1.90	+	3.28	+	4.18	+	0.38	−	0.32	−	0.29	−
Fibronectin	2.26	+	3.06	+	0.71		0.97		1.09		0.57	
Mmp7	0.56		8.08		41.93	+	26.43	+	9.26		53.13	+

Note. Each value represents the ratio of the mean value of the group treated with EE (3 μg/kg) to the mean value of the time-matched vehicle group; "+," significantly increase from the time-matched vehicle group ($p < 0.05$); and "−," significantly decrease from the time-matched vehicle group ($p < 0.05$).

to normalize the target mRNA expression in the uterus after treatment with ER agonist.

The expression of *Gapdh* mRNA normalized with 18S rRNA significantly increased between 3 and 24 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups (Fig. 2C). An increase of the *Gapdh* mRNA in the 3-μg/kg EE group reached a peak at 12 h (9.64-fold) after administration.

Effects of EE on the Expression of Sex Steroid Hormone Receptor Genes in the Uterus of Immature Female Rats

ERα, *ERβ*, progesterone receptor (*PR*), and androgen receptor (*AR*) mRNAs were selected to evaluate the effect of EE on the expression of sex steroid hormone receptor genes (Diel *et al.*, 2000; Waters *et al.*, 2001).

The expression level of *ERα* mRNA was about 100 times higher than that of *ERβ* mRNA in the uterus of immature rats. The expression of *ERα* mRNA significantly decreased from 24 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups and kept decreasing until 48 h (0.57-fold) (Fig. 3A, Table 3).

The expression of *ERβ* mRNA significantly increased at 1 h (2.01-fold) after treatment with 3 μg/kg EE, but thereafter it decreased more than those of the time-matched vehicle groups (the lowest: 0.25-fold at 6 h) (Fig. 3B, Table 3).

The expression of *PR* mRNA significantly decreased from 12 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups and kept decreasing until 48 h (the lowest: 0.40-fold at 24 h) (Fig. 3C, Table 3).

The expression of *AR* mRNA significantly increased at 3 h (1.93-fold) after treatment with 3 μg/kg EE as compared with that in the time-matched vehicle group but significantly decreased more than that of the time-matched vehicle group at 48 h (0.50-fold) (Fig. 3D, Table 3).

Since ERα, ERβ, PR, and AR are transcription factors to exert physiological functions specific to each ligand (Mangelsdorf *et al.*, 1995; Tasset *et al.*, 1990), it is suggested that estrogenic compounds may affect the expressions of their downstream target genes.

Effects of EE on the Expression of Well-Known Estrogen Target Genes in the Uterus of Immature Female Rats

Complement C3 (Diel *et al.*, 2000; Sundstrom *et al.*, 1989) and insulin-like growth factor 1 (*Igf1*: Klotz *et al.*, 2000) mRNAs were selected to evaluate the effect of EE on the expression of well-known estrogen target genes.

The expression of complement C3 mRNA significantly increased between 6 and 48 h after treatment with 3 μg/kg EE as compared with those in the time-matched vehicle groups