Transcriptome analysis reveals an unexpected role of a collagen tyrosine kinase receptor gene, Ddr2, as a regulator of ovarian function

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Submitted 21 April 2009; accepted in final form 10 August 2009

Matsumura H, Kano K, Marín de Evsikova C, Young JA, Nishina PM, Nagge1rt JK, Naito K. Transcriptome analysis reveals an unexpected role of a collagen tyrosine kinase receptor gene, Ddr2, as a regulator of ovarian function. Physiol Genomics 39: 120–129, 2009. First published August 11, 2009; doi:10.1152/physiolgenomics.00073.2009.—Mice homozygous for the smallie (slie) mutation lack a collagen receptor, discoidin domain receptor 2 (Ddr2), and are dwarfed and infertile due to peripheral dysregulation of the endocrine system of unknown etiology. We used a systems biology approach to identify biological networks affected by Ddr2slie/slie mutation in ovaries using microarray analysis and validate findings using molecular, cellular, and functional biological assays. Transcriptome analysis indicated several altered gene categories in Ddr2slie/slie mutants, including gonadal development, ovulation, antiapoptosis, and steroid hormones. Subsequent biological experiments confirmed the transcriptome analysis predictions. For instance, a significant increase of TUNEL-positive follicles was found in Ddr2slie/slie mutants vs. wild type, which confirms the transcriptome prediction for decreased chromatin maintenance and antiapoptosis. Decreases in gene expression were confirmed by RT-PCR and/or qPCR: luteinizing hormone receptor and prostaglandin type E and F receptors in Ddr2slie/slie mutants, compared with wild type, confirm hormonal signaling pathways involved in ovulation. Furthermore, deficiencies in immunohistochemistry for Ddr2 and luteinizing hormone receptor in the somatic cells, but not the oocytes, of Ddr2slie/slie mutant ovaries suggest against an intrinsic defect in germ cells. Indeed, Ddr2slie/slie mutants ovulated significantly fewer oocytes; their oocytes were competent to complete meiosis and fertilization in vitro. Taken together, our convergent data signify Ddr2 as a novel critical player in ovarian function, which acts upon classical endocrine pathways in somatic, rather than germline, cells.

discoidin domain receptor 2; dwarfism; infertility; luteinizing hormone receptor; mice; ovulation; prostaglandin receptor

Although collagen is primarily considered to be an extracellular matrix molecule that supports cell structure and architecture in bodily tissues and bone, it also may act as a signaling molecule, activating specific receptors at the cell surface (3, 9, 15, 27). Over the past decade, collagen has been identified and subsequently confirmed as an endogenous ligand for the two discoidin domain receptors (DDR; 4, 19, 22, 27, 50–52). DDR2 binds to and is activated by collagen I, II, III, V, and X, with the notable exception of basement membrane collagen IV (2, 28, 29, 43, 51). DDR2 is expressed in connective tissues arising from embryonic mesoderm, such as skeletal muscle, kidney, heart, lung, and ovary (4, 24, 26, 32). DDR2 regulates cell proliferation, cell adhesion, migration, as well as extracellular matrix remodeling (19, 26, 35, 55). Recently we characterized, mapped, and identified a spontaneous, homozygous recessive allele, smallie (slie), which contains a deletion in the gene for DDR2 (Ddr2) and causes dwarfism and infertility in mice (24).

The smallie allele, designated as Ddr2slie in genetic nomenclature, is a large intragenic deletion of ~150 kb encompassing exons 1–17 of Ddr2 gene, leaving its remaining exon 18 intact, along with its proximal gene neighbor (24). Thus, no DDR2 protein is synthesized in Ddr2slie/slie mice. At birth through weaning, Ddr2slie/slie mice are phenotypically indistinguishable from their wild-type littermates; however, the initial phenotype appears soon after weaning, as Ddr2slie/slie do not rapidly gain weight or exhibit a juvenile growth spurt, resulting in a disproportionate dwarfism with mild craniofacial abnormalities (24). The Ddr2slie/slie mice exhibit the same bone mineral density, but decreased total mineral content, compared with their wild-type siblings. While the dwarfism phenotype is not unexpected for a deletion of collagen receptor, given the pivotal functions of collagen for bone matrix formation and elongation (3, 9, 15), an infertility phenotype was not anticipated. Specifically, intercrossing young adult homozygous Ddr2slie/slie mice to other Ddr2slie/slie or heterozygous or wild-type mice failed to produce any progeny. Contrary to expectations, the dwarfism and infertility arise from a peripheral endocrine defect because the hypothalamic-pituitary hormonal axis was found to be intact and functional in Ddr2slie/slie mice (24). In fact, Ddr2slie/slie and control wild-type mice secrete similar levels of anterior pituitary hormones, contain a similar number and distribution of cells containing these hormones within the pituitary, and have comparable levels of mRNA for releasing hormones in the hypothalamus. However, gonadal steroid production is curtailed, even upon stimulation with exogenous pituitary hormones. Together, these data indicate a peripheral, not central, origin.

The purpose of this study was to ascertain cellular and molecular pathways affected by Ddr2 expression and signaling on ovarian function. To identify the molecules affected by the loss of DDR2 signaling in the ovary, we compared ovarian transcriptomes between Ddr2slie/slie mutants and wild-type mice using microarrays to measure relative gene expression across the entire mouse genome. Transcriptome analysis revealed a decrease in the relative expression of several gene
categories that likely regulate or affect reproduction in Drd2<sup>−/−</sup> mice compared with wild type. A subset of predictions derived from the transcriptome analysis and microarray gene expression data was subsequently tested within the context of biology using molecular, cellular, and physiological assays. Contrary to expectations, gonadotropin hormones increased DDR2 immunoreactivity in wild-type ovary, whereas exogenous gonadotropins did not induce luteinizing hormone receptor (LHR) gene expression or immunoreactivity in smallie ovaries, relative to wild-type control. Unexpectedly, DDR2 but did not alter matrix metalloproteinase (Mmp) gene expression in the ovary (12), which is the canonical signaling pathway for discoid domain receptors (34, 35). Furthermore, a decrease in LHR and (12), which is the canonical signaling pathway for discoid domain receptors (34, 35). Furthermore, a decrease in LHR and

**Materials and Methods**

*Animals.* Prepubertal and adult wild-type (BKS.HRS/J +/+) and smallie mice (BKS.HRS/J-Drd2<sup>−/−</sup>) were obtained by mating heterozygous mice carrying the Drd2<sup>−/−</sup> allele (24). All mice used in this study were bred either at the University of Tokyo or at The Jackson Laboratory, and all procedures in these studies were approved by their respective institutional Animal Care and Use Committees. Mice were housed in groups of two to four, with white pine shavings as bedding, under 12:12 h photoperiod (lights on at 07:00), with ad libitum access to water and food.

DNA microarray experiments. Total RNA was harvested from three adult, 10-wk-old wild-type or Drd2<sup>−/−</sup> mutant ovaries after gonadotropin treatment [5 IU of pregnant mare serum gonadotropin (PMSG) + 5 IU human chorionic gonadotropin (hCG) 48 h later] as separate samples using RNeasy Mini kit from QIAGEN (Valencia, CA) according to the manufacturer’s protocol. We used the small-scale protocol to reproducibly amplify in a linear manner and label total RNA (Affymetrix, Santa Clara, CA). Gene expression was analyzed using GeneChip Mouse Genome 430 2.0 Array covering >39,000 transcripts on a single array (Affymetrix). The procedures of converting RNA to cDNA, labeling, microarray hybridization, and GeneChips scanning were performed at the Microarray Facility at the University of Tokyo (Tokyo, Japan) as described in the Affymetrix protocol. The microarray data have been submitted to the Center for Information Biology gene expression database (accession no. CBX83).

**Transcriptome analysis.** Prior to computational analyses, quality control measures were performed on the array data. For each differentially expressed Affymetrix probe set, a corresponding Mouse Genome Informatics (MGI) gene accession number was identified by screening against the ENSEMBL mouse genome assembly (http://www.ensembl.org/Mus_musculus, version 49.37b). This was done to verify that probe sets indeed unambiguously recognized “authentic” genes. Probes that identified retrotransponson sequences and probes mapping to multiple genes were discarded, because these often produce ambiguous and even false gene expression data. In addition, when multiple probe sets for the same gene gave contradictory or opposing signals, the corresponding gene was discarded from further analysis.

**VisuAL annotation display analysis.** VisuAL Annotation Display (VLAD) software, developed by the MGI staff (7, 8), searches existing Gene Ontology (GO) annotations (5) for a designated query set of genes. The GO project is a collaborative effort that assigns consistent descriptions for gene products within three structured controlled vocabularies (ontologies: biological processes, cellular components, and molecular functions) for plant, animal, and microbial genomes, in terms of their associated functions in a species-independent manner (5). VLAD software analyzes the data and displays a summary, in graphical and tabular forms, of the GO classifications relevant to the query. VLAD software calculates the hypergeometric distribution statistic and can be used to discover statistically significant overrepresentation of distinct GO categories within three major controlled vocabularies in the query set relative to a specified “universe” gene set. After quality control, microarray probe IDs were matched to their respective MGI accession numbers, and these were used as a query set to compare against all mouse genes, which were set as the universe the analysis.

**Reverse-transcriptase PCR and quantitative real-time PCR.** For semiquantitative RT-PCR, total RNAs were isolated from whole ovaries from wild-type (n = 3) and Drd2<sup>−/−</sup> mutant (n = 5) mice using TRIzol reagent (Invitrogen Life Technologies, Carlsbad, CA). cDNA was synthesized from total RNA with Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). The primers used to detect genes Drd2, Mmp2 (matrix metallopeptidase 2), Mmp9 (matrix metallopeptidase 9), Mmp19 (matrix metallopeptidase 19), Mmp14 (matrix metallopeptidase 14), Lhcgr (luteinizing hormone/choriogonadotropin receptor), Fshr (follicle stimulating hormone receptor), Ptger2 (prostaglandin E receptor 2, subtype EP2), Ptgfr (prostaglandin F receptor), and housekeeping gene Gapdh (glyceraldehyde-3-phosphate dehydrogenase, also known as G3PDH) are listed in Table 1. PCR was performed with AccuPrime PfX DNA Polymerase (Invitrogen). The amplification conditions used were 98°C, 30 s followed by 15–25 cycles of 98°C, 10 s, 58°C, 30 s; 72°C, 30 s. The number of cycles used ensured that the reaction could be quantified within the log phase of the amplification reaction.

**Table 1. Oligonucleotide primer sequences used for RT-PCR or qPCR**

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<thead>
<tr>
<th>Gene</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
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<tr>
<td>Drd2</td>
<td>GTCCTCACAGTTGTCCTAGATG</td>
<td>GGATCAAGCGACTCGATGAC</td>
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<td>Mmp2</td>
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<td>CCACTGCGATCTGAGATC</td>
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<td>Mmp9</td>
<td>CTTCAAGAGGCTGTTGAGTGCTG</td>
<td>GGATGATGCTGTTGAGATC</td>
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<td>Mmp19</td>
<td>GTCTCAGTACGTTGAGATGCTG</td>
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</tr>
<tr>
<td>Mmp14</td>
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<td>CCACTGCGATCTGAGATC</td>
</tr>
<tr>
<td>Lhcgr</td>
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<td>GGATCAAGCGACTCGATGAC</td>
</tr>
<tr>
<td>Fshr</td>
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<td>CCACTGCGATCTGAGATC</td>
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<tr>
<td>Ptger2</td>
<td>CCGACAGGCACTCCGGAAGAGAGGACG</td>
<td>CCACTGCGATCTGAGATC</td>
</tr>
<tr>
<td>Ptgfr</td>
<td>TCTTTTCCCGAGGAGAGAGGAGGAGGACG</td>
<td>CCACTGCGATCTGAGATC</td>
</tr>
<tr>
<td>Gapdh</td>
<td>TGAAGGCGGTGCCGGAAGAGAGGAGGACG</td>
<td>CCACTGCGATCTGAGATC</td>
</tr>
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Drd2, discoidin domain receptor family, member 2; Mmp2, matrix metallopeptidase 2; Mmp9, matrix metallopeptidase 9; Mmp14, matrix metallopeptidase 14; Lhcgr, luteinizing hormone/choriogonadotropin receptor; Fshr, follicle stimulating hormone receptor; Ptger2, prostaglandin E receptor 2, formerly EP2; Ptgfr, prostaglandin F receptor; Pgr, progesterone receptor; Gapdh, glyceraldehyde-3-phosphate dehydrogenase.

*Physiol Genomics* • VOL 39 • www.physiolgenomics.org
For quantitative real-time PCR (qPCR), total RNAs were isolated using TRIzol reagent from the whole ovaries of wild-type (n = 3) or Ddr2^slie/slie (n = 3) mice after treatment with 10 IU PMSG ip followed 48 h later by 10 IU hCG ip (Invitrogen Life Technologies). cDNA was synthesized from total RNA with Superscript First-Strand Synthesis System for RT-PCR (Invitrogen). The amplification conditions used were 95°C, 2 min, followed by 25 cycles of 94°C, 15 s; 58°C, 30 s; 72°C, 30 s. The expression level [threshold cycle (Ct)] of each transcript was detected by counting the number of necessary cycles to yield equivalent expression level of each gene. Ct values were converted to fold differences in expression according to the equation 2^([Ct]_18s rRNA) - [Ct2(–/+)] - [Ct2(18s rRNA)], where Ct1(18s rRNA) and Ct2(18s rRNA) represent the Ct values for the 18s rRNA gene in the slie and wild-type samples, respectively. The same primers used for RT-PCR were used for qPCR, along with Pgr (progesterone receptor), with the exception of Mmp14 and Mmp19, which failed to yield a reliable signal for qPCR and was excluded from analysis.

Histology, immunohistochemistry, and TUNEL staining. In morphological experiments, 5 IU of PMSG (Serotropin; Aska Pharmaceutical, Tokyo, Japan) followed 48 h later by 5 IU hCG (Gonatropin; Aska Pharmaceutical) were injected ip into 10-wk-old wild-type male mice at 37°C and 5% CO2 in air for 4 h. Oocytes were processed (data not shown). All the reactions were carried out at room temperature. TUNEL staining was performed according to the manufacturer’s protocol (DeadEnd Colorimetric TUNEL System; Promega, Madison, WI).

Western blotting. Total protein was isolated from whole ovary of wild-type and Ddr2^2^slie/mutant mice. The protein samples were separated by electrophoresis using a 10% sodium dodecyl sulfate polyacrylamide gel. Proteins were transferred to a polyvinylidene fluoride membrane (AE-6660; Atto, Tokyo, Japan). After blocking the membrane with 3% skim milk for 20 min, we incubated the membrane with 1:5,000 diluted anti-goat IgG antibody conjugated to horseradish peroxidase (HRP) complex (Vectastain Elite ABC, Vector Laboratories) for 30 min. As a negative control, sections without the primary antibody were processed (data not shown). All the reactions were carried out at room temperature. TUNEL staining was performed according to the manufacturer’s protocol (DeadEnd Colorimetric TUNEL System; Promega, Madison, WI).

Ovulation and in vitro fertilization. To induce ovulation, 12–14-wk-old wild-type (n = 8) and Ddr2^2^slie/mutant (n = 4) female mice were injected (ip) with 5 IU PMSG and 48 h later with 5 IU hCG. Oviducts were removed 14 h after hCG injection to collect ovulated oocytes. Collected oocytes were incubated with capacitated sperm from ICR males at 37°C and 5% CO2 in air for 4 h. Oocytes were removed, rinsed from sperm, and placed into fresh media. After incubation for 20 h at 37°C and 5% CO2 in air, two-cell embryos were transferred to fresh M16 medium (Sigma) to develop in vitro for 3 days until the expanded blastocyst stage.

Statistical analysis. Descriptive statistics (means and SE) were reported for number of ovulation oocytes in female wild-type and Ddr2^2^slie/mutant mice. To detect significant statistical differences, nonparametric Mann-Whitney or parametric Student’s t-tests were used to compare among experimental groups. Differences were considered significant at P < 0.05 (Statview software; SAS Institute, Cary, NC). For VLAD analysis, hypergeometric distribution tests were used to determine significant differences in GO categories (P < 0.05).

RESULTS

Transcriptome analysis of ovarian gene expression. To circumvent technical bias and unreliable data interpretation, the quality control of microarray data is necessary and essential to yield a reliable analysis of gene expression. It is vitally important in studies involving whole tissues, which by nature contain heterogeneous cell types, such as ovaries. Using the quality control procedures described in the methods, 117 Affymetrix probe sets were discarded, leaving a total of 2,519 probes representing 2,049 expressed genes. Sixty-eight of these probes did not correspond to genes, and most recognizing retrotransposons rather than genes. Comparing Ddr2^2^slie/mutants and wild-type gene expression among the remaining quality-controlled set of probes, ~47% genes had a relative increase in expression levels, whereas ~52% had a relative decrease in expression. Statistical analysis confirmed an underlying random distribution of genes expressed in germ cells (oocytes) vs. whole ovary when the microarray data were matched against the large representative dataset of known fully-grown oocyte transcripts (18; χ² = 0.18, P > 0.67). Thus, the quality control procedures yielded a large data set of gene expression appropriate for transcriptome analysis of heterogeneous ovarian tissue because it was statistically shown to be free of bias toward either somatic or germ cell types.

To identify the major functional classes of all differentially expressed genes in Ddr2^2^slie/mutants, the VLAD tool for extraction and analysis of GO annotations for large sets of genes was used (7, 8). VLAD analysis revealed perturbations in genes expressed in ovary that belong to major GO categories, such as cell death, inflammatory response, cholesterol biosynthesis and development and differentiation (Fig. 1A). Notably, a narrow set of biologically related gene classes were relatively increased in Ddr2^2^slie/mutants, compared with wild type; all of which involved inflammation and immune processes, such as cytokine pathways, antigen production, and inflammatory responses (Fig. 1B). In contrast, the classes of genes that showed a relative decreased expression in Ddr2^2^slie/mutants included many classical molecular and cellular pathways known to be involved with ovarian function and regulation, such as antiapoptosis, steroid synthesis, gonadal development, ovolatory cycles, and ovulation (Fig. 1C). Furthermore, decreases in the relative gene expression of antiapoptosis genes corroborated with decreases in other categories, of chromatin maintenance and assembly, as well as DNA packaging genes, which attests to the integrity of the transcriptome data analysis (Fig. 1C). Many of these molecular pathways potentially underlie infertility in Ddr2^2^slie/mutants. To confirm genes in these categories are contributing to infertility in Ddr2^2^slie/mutants, we conducted several cellular,
molecular, histological, and physiological experiments to confirm the predictions from transcriptome analysis.

Confirming DDR2 regulation of ovarian cyclicity and ovulation via gonadotropin hormones. To confirm the prediction from transcriptome analysis that a regulatory feedback for ovulation exists between gonadotropins and DDR2, we tested whether gonadotropin stimulation induces follicular growth and ovulation in adult Ddr2<sup>slie/slie</sup> mutants relative to wild type. To determine if gonadotropins regulate DDR2 levels in the ovary, PMSG and hCG were injected into wild-type and Ddr2<sup>slie/slie</sup> mutant female mice. According to indirect immunohistochemistry, DDR2 immunostaining was moderately elevated after PMSG compared with saline alone (Fig. 2A and B); however, a larger increase occurred after administration of both PMSG and hCG, most notably in somatic cells, such as interstitial and thecal cells (Fig. 2C) but not in the germ cells (i.e., oocytes). Conversely, as expected, DDR2 immunostaining for DDR2 (beyond background signal) was not detected in Ddr2<sup>slie/slie</sup> mutants after exogenous gonadotropin stimulation (Fig. 2, D–F). In addition, these observations of increased DDR2 protein content after gonadotropin treatment from immunohistochemistry experiments were confirmed by immunoblot studies of whole ovary extracts from wild-type mice (Fig. 2, G and H). These data confirm that a regulatory loop exists between DDR2 and gonadotropins.

Ovulation and embryonic development. To verify, as predicted by transcriptome analysis, the suppression of gonadal development and ovulation-related genes, but not fertilization and embryo gene categories, we examined ovulation induced by exogenous gonadotropin stimulation and oocytes’ potential to complete fertilization and embryonic development in vitro. The number of oocytes ovulated after the superovulation was significantly decreased in adult Ddr2<sup>slie/slie</sup> mutant mice (Fig. 3A). To investigate whether oocytes from Ddr2<sup>slie/slie</sup> mutant mice are competent to complete meiosis, fertilization, and development, we performed in vitro fertilization with oocytes from Ddr2<sup>slie/slie</sup> mutant mice and cultured the fertilized oocytes in vitro. One-cell zygotes did develop to the expanded blastocyst stage (Fig. 3B). Although substantially fewer oocytes were ovulated, these oocytes proved to be competent to complete fertilization and early embryogenesis.

Apoptosis in Ddr2<sup>slie/slie</sup> mutant ovaries. As indicated by transcriptome analysis, many antiapoptotic genes were relatively suppressed in Ddr2<sup>slie/slie</sup> mutant ovaries (Fig. 1, A and C; Supplemental Table S11) and suggest that an increase in cell death occurs in mice lacking Ddr2. To test the prediction that a greater incidence of follicular apoptosis occurs in Ddr2<sup>slie/slie</sup> mutant ovaries, we processed ovarian sections for TUNEL assays to illustrate an increase in DNA breakage, a hallmark of programmed cell death, in adult wild-type (Fig. 4, A and B) and Ddr2<sup>slie/slie</sup> mutant mice (Fig. 4, C and D). Significantly more apoptotic cells were observed in the follicles of 10-wk-old Ddr2<sup>slie/slie</sup> mutant compared with wild-type ovaries (Fig. 4E).

Hormonal and molecular pathways affected by Ddr2. The results from ovarian transcriptome analysis (Fig. 1), the primary microarray data (Supplemental Table S1), along with the data from the immunohistochemistry experiments and published scientific knowledge (1, 6, 12, 17, 23, 31, 36, 39, 42, 55)
informed our decision to focus on a subset of genes for gene expression studies. To confirm a subset of prominent molecules regulating ovulation as identified by transcriptome analysis (Fig. 1), by primary expression data by microarray (Supp. Table 1), and by previous published studies, we assessed expression levels of genes Ddr2, Mmp2, Mmp9, Mmp14, Mmp19, Lhcg, Pgr, Ptger2, Ptgsr, and housekeeping gene Gapdh mRNAs by either RT-PCR or qPCR (or both techniques) following administration with PMSG and hCG. As expected, the expression levels of Ptgsr, Lhcg, and Ptger2 genes were statistically significantly lower in Ddr2s/sie ovaries relative to wild type (Fig. 5, A and B). Among these downregulated genes, Lhcg is known to have important role in ovulation through luteinizing hormone signaling. To assess the alteration of expression and localization after exposure to exogenous gonadotropins, we performed indirect immunohistochemistry for LHR. We observed fewer immunopositive cells for LHR after saline (Fig. 5F), as well as after exogenous PMSG + hCG administration, especially in interstitial cells of the Ddr2s/sie ovaries at 3 wk of age compared with wild type (Fig. 5G).

Contrary to our expectation, transcriptome analyses using VLAD software and primary microarray data, did not predict any significant changes in expression levels for genes Pgr, Fshr, or in two members of the matrix metalloproteinase (Mmp2, Mmp9) family, the canonical downstream pathway activated by DDR2 in somatic cells (7, 8), which are also expressed in ovarian thecal cells (12). To demonstrate a lack of disruption in these pathways in Ddr2s/sie mutant ovaries, RT-PCR or qPCR experiments were conducted to verify the absence of differential expression. The expression levels of these genes were not significantly different between Ddr2s/sie mutant and wild-type mice in response to gonadotropin stimulation, which further supports the predictions from transcriptome analysis (Fig. 5, A–C).

**DISCUSSION**

Transcriptome analysis was statistically performed for over-representation of gene categories involved with reproduction, apoptosis and programmed cell death, and inflammation in Ddr2s/sie mutants compared with wild-type mice. Next, we verified experimentally whether a subset of these gene categories indicated by transcriptome analysis was indeed altered, and the biological significance of this alteration. Treatment with exogenous gonadotropin hormones increased DDR2 immunoreactivity in wild-type ovary, whereas no DDR2 immunoreactivity was detected in ovarian tissue from Ddr2s/sie mutants. In a similar pattern, exogenous gonadotropin administration did not induce LHR immunoreactivity in ovarian tissue from Ddr2s/sie mutants relative to wild-type control. Furthermore, gene expression levels were statistically significantly decreased in the genes encoding Lhcg, Ptger2, and Ptgsr in ovaries from Ddr2s/sie mutants, relative to wild-type mice, as detected by either RT-PCR or qPCR (or both). Gene expression levels for Mmp genes in ovarian tissue from Ddr2s/sie mutants did not statistically significant differ from wild-type mice, as measured by RT-PCR and qPCR, which was unexpected as Mmp activity is currently considered the canonical signaling pathway for discoidin domain receptors (12, 34, 35). In summary, these convergent results suggest that DDR2 may ultimately affect fertility in female mice through altering gene expression for classical endocrine pathways to regulate ovarian function.

Ovulation is a complex process initially triggered by LH that culminates in the release of an oocyte, competent to complete

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**Fig. 2.** Gonadotropin regulation of DDR2 expression in ovaries. DDR2 indirect immunoreactivity (brown) in cross-sections of ovaries from adult wild type (+/+) and Ddr2s/sie mutant, counterstained with hematoxylin (violet), after saline (A, D), PMSG (B, E), or PMSG + hCG (C, F) treatments. DDR2 immunoreactivity was elevated after PMSG alone compared with saline and especially after PMSG + hCG in interstitial cells (open arrowheads) of the ovaries (B, C), whereas ovarian sections from Ddr2s/sie mutants served as negative control (E, F). Immunoblot (G) and graph of relative optical density for LHR immunoreactive protein (H) harvested from extracts of whole ovaries from wild type (+/+) and Ddr2s/sie mutants. In a similar pattern, exogenous gonadotropin administration did not induce LHR immunoreactivity in ovarian tissue from Ddr2s/sie mutants. In a similar pattern, exogenous gonadotropin administration did not induce LHR immunoreactivity in ovarian tissue from Ddr2s/sie mutants relative to wild-type control. Furthermore, gene expression levels were statistically significantly decreased in the genes encoding Lhcg, Ptger2, and Ptgsr in ovaries from Ddr2s/sie mutants, relative to wild-type mice, as detected by either RT-PCR or qPCR (or both). Gene expression levels for Mmp genes in ovarian tissue from Ddr2s/sie mutants did not statistically significant differ from wild-type mice, as measured by RT-PCR and qPCR, which was unexpected as Mmp activity is currently considered the canonical signaling pathway for discoidin domain receptors (12, 34, 35). In summary, these convergent results suggest that DDR2 may ultimately affect fertility in female mice through altering gene expression for classical endocrine pathways to regulate ovarian function.

Ovulation is a complex process initially triggered by LH that culminates in the release of an oocyte, competent to complete
meiosis and initiate embryogenesis after fertilization (38, 39, 41). Coordination of autocrine, paracrine, and endocrine pathways affecting associated remodeling of ovarian tissue is essential and critical. To date, little is known about the exact role of extracellular matrix molecules, specifically collagen and its receptors, in ovulation and follicular development. For ovarian pathology and disease, it is known that signaling molecules for the extracellular matrix, specifically members of the \textit{Mmp} gene family, may be involved with rendering metastatic cancer cells the ability to infiltrate the ovarian epithelium, and thus may be a useful biomarker for diagnosis of ovarian cancer (1, 4, 44, 45, 50, 54). Collagen has been recognized as a component of ovarian tissue in mammals, but it is also known, in other nonmammalian vertebrate species, such as trout, that collagen is present in the ovary (53). It has been proposed that the extracellular matrix, namely collagen, and presumably its receptors, participates in ovarian function as molecule facilitating remodeling of ovarian tissue, which is required for successful ovulation of mature oocytes (49). However, the details about ligands, molecules, receptors, and intracellular signaling pathways of the extracellular matrix involved in ovulation and remodeling of ovarian tissue are not known. Here we show that one of the potential extracellular matrix molecules participating in this process is DDR2.

DDR2 is a fibrillar collagen receptor widely expressed in various tissues and participating in cellular proliferation, adhesion, migration, and tissue remodeling (19, 26, 35, 55). Furthermore, DDR2 is a recently discovered molecule that appears to have a critical role in reproduction, as mice with a homozygous, autosomal deletion of the \textit{Ddr2} gene are dwarfed and infertile (24). In addition, humans carrying a homozygous, missense mutation causes SMED-SL, manifesting as disproportionate dwarfism with shortening of the long bones, mild craniofacial abnormalities, and infertility (6). In this current study, we focused on the molecular and cellular pathways underlying infertility in female \textit{Ddr2}\textsuperscript{slie/slie} mutant mice. Specifically, as a first step to understand how deficient DDR2 signaling leads to an infertility phenotype, we applied bioinformatics tools to analyze global gene expression data from DNA microarrays to identify molecular and cellular pathways in ovarian tissue affected by a loss-of-function allele for \textit{Ddr2}, known as \textit{smallie}, compared with wild-type allele, in mice. In summary, we tested a subset of the predictions from our transcriptome analysis with further in vivo and in vitro biolog-

\begin{figure}[h]
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\includegraphics[width=\textwidth]{fig3}
\caption{Ovulation and developmental capacity of \textit{Ddr2}\textsuperscript{slie/slie} oocytes. A: significantly fewer oocytes were ovulated in \textit{Ddr2}\textsuperscript{slie/slie} mutant compared with wild type (+/+ ) after injection with PMSG + hCG. B: some in vitro fertilization oocytes from \textit{Ddr2}\textsuperscript{slie/slie} mutant mice developed from one-cell to blastocyst. * \textit{P} < 0.01.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{Follicle cell death in ovaries from \textit{Ddr2}\textsuperscript{slie/slie} mutants. Histological sections of ovaries from wild-type (+/+ ) and \textit{Ddr2}\textsuperscript{slie/slie} mutant at 10 wk stained with hematoxylin and eosin (A, C), and TUNEL analyses of ovaries (B, D) after treatment with PMSG + hCG. Higher counts of apoptotic follicles were observed in \textit{Ddr2}\textsuperscript{slie/slie} mutant mice compared with wild-type per ovarian section (E). HE, hematoxylin and eosin; TUNEL, terminal deoxynucleotidyltransferase-mediated deoxyuridine triphosphate nick end labeling. * \textit{P} < 0.01; Scale bar, 200 \textmu m.}
\end{figure}
ical experiments, including quantitative RT-PCR, immunohis-
tochemistry, histological, and physiological studies. These ex-
perimental biological approaches confirmed that bioinformat-
icos analyses are a robust "systems biology" tool to identify
specific molecular pathways, especially in the perplexity of
large gene expression datasets.

The goal of any microarray experiment is to identify the
genes that are differentially expressed any samples to yield
insight into the complex pathways involved with biological
processes. Unfortunately, generating a list of genes by employ-
ing an arbitrary relative expression change cutoff value,
although still in current use, fails to provide any robust measure
of confidence for those genes selected and yields the false
positive selection of genes more susceptible to technical arti-
facts and experimental noise than actual expression changes
(10, 11, 14). To circumvent this widespread caveat in microar-

### Table: Gene Expression Confirmation

<table>
<thead>
<tr>
<th>Genes</th>
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<tr>
<td>Mmp2</td>
<td>Increased</td>
</tr>
<tr>
<td>Mmp9</td>
<td>Increased</td>
</tr>
<tr>
<td>Mmp19</td>
<td>Increased</td>
</tr>
<tr>
<td>Mmp14</td>
<td>Increased</td>
</tr>
<tr>
<td>Gapdh</td>
<td>Unchanged</td>
</tr>
</tbody>
</table>

Fig. 5. Gene expression confirmation studies in ovarian tissue from wild-type (+/+) and Ddr2<sup>slie/slie</sup> mutant mice. Gene expression is affected in Ddr2<sup>slie/slie</sup> mutant ovaries at 10 wk of age after gonadotropin treatment. Reverse-transcriptase PCR (A, B) and qPCR (C) analysis confirmed some genes identified from transcriptome analysis: Ddr2, Mmp2 (matrix metalloproteinase 2), Mmp9 (matrix metalloproteinase 9), Mmp19 (matrix metalloproteinase 19), Mmp14 (matrix metalloproteinase 14), Lhcgr (luteinizing hormone/choriogonadotropin receptor), Fshr (follicle stimulating hormone receptor), Ptger2 (prostaglandin E receptor 2, subtype EP2), Ptgfr (prostaglandin F receptor), Pgr (progesterone receptor), and housekeeping gene Gapdh (glyceraldehyde-3-phosphate dehydrogenase, also known as G3PDH) in wild-type and Ddr2<sup>slie/slie</sup> mutant ovaries after injection with PMSG and hCG. All data represent means and SE. Significant differences indicated by *<sup>P</sup> < 0.05, **<sup>P</sup> < 0.01. D–G: LHR (brown) protein levels were examined by indirect immunohistochemistry of ovarian tissue from wild type and Ddr2<sup>slie/slie</sup> mutant after saline (D, F), and PMSG + hCG (E, G) stimulation. LHR immunoreactivity was decreased in interstitial cells of the Ddr2<sup>slie/slie</sup> ovaries (arrowheads) after treatment to either saline alone or exogenous gonadotropins (F and G). O, oocytes. Scale bar 100 μm.
ray studies, we employed the use of publicly available bioinformatics databases, such as GO, and associated analysis platforms, such as VLAD, to examine the relationships of all differentially expressed genes, instead of focusing on a handful of essentially arbitrary chosen “candidate” genes. Indeed, a simple list of differentially expressed genes has two distinct disadvantages: lack of the insight into the complexity of fundamental biological processes, and the human bias due to the experimentalists’ temptation to focus on a handful of “favorite” genes. Furthermore, to contain the false discovery under 5% in our VLAD-based transcriptome analysis, we discarded categories of GOs with fewer than five genes members, since inclusion of even only one gene of those members would artificially yield a higher significance in those categories due to few observations (14, 21, 25). Applying these conservative constraints to our microarray data, transcriptome analysis did yield significant insights into pathways affected by disrupting DDR2 signaling in ovarian tissue and led to experimentally testable predications on the pathways indicated by VLAD analysis.

For example, VLAD indicated that the infertility phenotype in Ddr2slie/slie mutant mice may manifest as curtailed ovulation, increased follicular apoptosis, impaired hormone secretion and decreased follicular luteinization. Although cell death is clearly involved, we interpret these data that apoptosis is not the initial trigger underlying female infertility in Ddr2slie/slie mutant mice. Our interpretation is substantiated by experiments demonstrating that absence of DDR2 signaling does not affect apoptosis in other somatic tissues, such as chondrocytes or corneal fibroblasts (26, 32). We interpret these results that DDR2 does not provoke apoptosis directly; instead, apoptosis is a downstream consequence of alteration in other pathways due to the lack of robust DDR2 signaling, such as hormonal signaling via molecules like LHR. Indeed, mice homozygous for null alleles of Lhcgr, liver receptor homolog I (Lrh1) and prolactin receptor also display impaired fertility, ovulation and increased follicular apoptosis, a similar phenotype observed in our current and recent studies with Ddr2slie/slie mutant mice (16, 37).

While the specific role of ovarian theca cells is obscure, their putative role in proteolytic events of follicle rupture is undoubtedly critical for ovulation. The extracellular matrix within the theca layer should be altered upon gonadotropin signals to be digested at the ovarian surface (17). This is the first report that documents DDR2 levels are increased by exogenous gonadotropin stimulation in 3-wk-old Ddr2slie/slie mutant mice (20, 48) and dissimilarity of PTGER2 and DDR2 expression patterns (42) suggest that insufficient PTGER2 might not initiate infertility of Ddr2slie/slie mutant mice. Finally, the localization of LHR on the external theca cells corresponds to, and overlaps with DDR2 expression (31). In addition, the ovarian phenotypes of Ddr2slie/slie mutant mice are similar to genetically engineered mice, homozygous for Lhcgr null allele (36, 56). However, unlike Lhcgr null mutants, Ddr2slie/slie mutants develop deficiencies in ovarian function gradually, during the transition from juvenile to adulthood, and Ddr2slie/slie mutants do retain baseline Lhcgr expression, at least at puberty. The exact molecular and cellular mechanisms and pathways transducing LHR signals to DDR2 are not yet identified, but we speculate that DDR2 may possibly affect somatic cell proliferation during the LH surge. It is also possible that DDR2 and LHR may positively co-regulate each other to gradually induce remodeling of the follicular wall and release of the oocyte.

To summarize, Ddr2 expression is increased by exogenous gonadotropin in wild type, but not Ddr2slie/slie mutant, ovaries. Surprisingly, DDR2 signaling pathways did not activate Mmp genes, as no differences in their expression were found in Ddr2slie/slie mutants compared with wild-type mice. Instead, our findings suggest that overlapping spatial and temporal expression of DDR2 and LHR may permit their mutual co-regulation during specific periods of ovulation. Our results support the interpretation that a lack of DDR2 signals in Ddr2slie/slie mutants most likely triggers anovulation by altering gene expression of Lhcgr, and to a lesser extent, Ptgfr and Ptger2. Reduced expression of these receptors, in particular Lhcgr, may lead to subsequent down regulation of anti-apoptosis genes arising from impaired hormone signaling, and in turn drive follicular apoptosis, anovulation, and ultimately infertility in Ddr2slie/slie mutants.

ACKNOWLEDGMENTS

Our gratitude is extended to Alexei Evsikov for critique of the manuscript.

GRANTS

The research was supported in part by Grants-in-Aid for Scientific Research from Ministry of Education, Science, Sports, and Culture of Japan; the Morinaga Foundation; Foundation for Growth Science (K. Kano, H. Matsunuma, K. Naito); and National Institute of Diabetes and Digestive and Kidney Diseases Grants DK-46977 and DK-73267 (to C. Marin de Evsikova and J. K. Naggert).
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Physiol Genomics • VOL 39 • www.physiolgenomics.org