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Discoidin Domain Receptor 1 Tyrosine Kinase Has an Essential Role in Mammary Gland Development

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Various types of collagen have been identified as potential ligands for the two mammalian discoidin domain receptor tyrosine kinases, DDR1 and DDR2. Here, we used a recombinant fusion protein between the extracellular domain of DDR1 and alkaline phosphatase to detect specific receptor binding sites during mouse development. Major sites of DDR1-binding activity, indicative of ligand expression, were found in skeletal bones, the skin, and the urogenital tract. Ligand expression in the uterus during implantation and in the mammary gland during pregnancy colocalized with the expression of the DDR1 receptor. The generation of DDR1-null mice by gene targeting yielded homozygous mutant animals that were viable but smaller in size than control littermates. The majority of mutant females were unable to bear offspring due to a lack of proper blastocyst implantation into the uterine wall. When implantation did occur, the mutant females were unable to lactate. Histological analysis showed that the alveolar epithelium failed to secrete milk proteins into the lumen of the mammary gland. The lactational defect appears to be caused by hyperproliferation and abnormal branching of mammary ducts. These results suggest that DDR1 is a key mediator of the stromal-epithelial interaction during ductal morphogenesis in the mammary gland.

Membrane-bound receptors with intrinsic protein tyrosine kinase activity are designed to sense a variety of extracellular stimuli. Activated receptor tyrosine kinases (RTKs) initiate signaling pathways leading to proliferation, differentiation, metabolism, survival, or cell death. It has been estimated that mammalian cells contain at least 150 genes coding for protein tyrosine kinases (13).

The two discoidin domain receptors, DDR1 and DDR2, represent a subfamily of RTKs and are expressed in a variety of mouse and human tissues (34). In the N-terminal part of the extracellular region, DDRs have a region related to the *Dictyostelium discoideum* protein discoidin. During cell aggregation of *Dictyostelium*, discoidin is secreted and functions as a lectin. In higher organisms, discoidin domains have been recognized in a variety of membrane-bound and secreted proteins, such as the neuropilins or blood clotting factors V and VIII (4). However, it still remains to be shown that mammalian discoidin domains have a binding affinity to carbohydrates.

Various types of collagen have been identified as potential ligands capable of activating both DDRs. Whereas DDR1 autophosphorylation is induced by all collagens so far tested (type I to type VI), DDR2 is only activated by fibrillar collagens, in particular by collagen type I and type III (30, 33). In contrast to most other RTKs, DDR activation by collagen follows slow kinetics and can take up to 18 h to reach maximal kinase activity. While it is apparent that collagen needs to be in its native, triple-helix configuration to activate DDR, the binding epitopes for the DDR extracellular domain on a collagen molecule and the significance of the discoidin region in collagen binding are not yet defined (30, 33). It remains entirely possible that DDRs bind additional ligands, which might act together with or separately from collagen.

While the biological function of DDR1 is unknown, its expression pattern has been analyzed in a variety of normal and malignant tissues. The DDR1 cDNA has been isolated from several human carcinoma cells, notably from MCF7 mammary carcinoma cells, ovarian and esophageal cancer cells, HeLa cells, and primary pediatric brain tumor samples, and also from human keratinocytes (1, 9, 14, 17, 22, 36). Genes homologous to human DDR1 have been identified in the mouse and rat (29, 37) and in invertebrates. Mammalian DDR1 mRNA is highly expressed in kidney, lung, thyroid, and brain (1, 29, 37). RNA in situ hybridization analysis has shown specific expression of human DDR1 in epithelial cells, for example in the mucosa of the colon, the follicles of the thyroid, and the islets of Langerhans (1). During mouse pregnancy, an increase in DDR1 mRNA transcripts in the mammary gland has been detected (3). Furthermore, DDR1 is significantly overexpressed in several human breast tumors (3, 23). The DDR1 promoter displays a potential p53 binding site, and DDR1 expression can be up-regulated by expression of p53 in human osteosarcoma cells (28). The expression of DDR1 and its ligands in the cerebellum has been analyzed in more detail (5). In the cerebellum, dominant-negative mutants of DDR1 blocked the elongation of granule neurones.

Thus far, three isoforms of the DDR1 protein that arise by alternative splicing have been characterized. The longest DDR1 transcript codes for the full-length receptor (c-isoform), whereas the a- or b-receptor isoforms lack 37 or 6 amino acids in the juxtamembrane or kinase domain, respectively (1). The

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phosphotyrosine-binding domain of the adapter protein ShcA interacts with an LLXNPXY site located in the alternatively spliced insert of DDR1b (33). The juxtamembrane region of DDR1a, transfected into PC12 cells, interacts with the FRS2, a protein adapter which has been identified as a substrate of the fibroblast growth factor receptor (11). Constitutive tyrosine phosphorylation of DDR1 was reported in T-47D cells, possibly due to endogenously secreted collagen proteins (14). Exogenous collagen type I and type V induce a substantial increase of DDR1 tyrosine kinase activity in T-47D cells (33).

In this study, we have identified DDR1-binding sites by staining mouse sections with the extracellular domain of DDR1 conjugated with alkaline phosphatase. To analyze the in vivo role of DDR1, we have introduced a deletion into the DDR1 gene in the mouse germ line. Mice lacking DDR1 are small, and mutant females show multiple reproductive defects. The majority of females are unable to give birth because developing blastocysts do not implant. Successfully reproducing females are unable to nourish their litters because the mammary gland epithelium fails to secrete milk.

MATERIALS AND METHODS

Vector and plasmid constructs. A genomic library from 129/Sv mice was probed with a cDNA fragment coding for the alternatively spliced exon 11 of DDR1 (24, 28). From two overlapping genomic clones, the targeting vector was constructed using the pPNT plasmid (32). To do this, a 750-bp *Eco*RI/*Bam*HI fragment and a 2.3-kb *Xho*I/*Not*I fragment were isolated and cloned to either side of the neo^r cassette. In the mouse DDR1 locus, the *Eco*RI site is located in the 5′-untranslated region and the *Bam*HI site is 12 bp upstream of the start codon. The *Xho*I site is located in the 12th intron, and the *Not*I site is in the last coding exon. The linearized vector was electroporated into R1 embryonic stem (ES) cells, and transfected cells were selected with G418 and ganciclovir. DNA from drug-resistant colonies was digested with *Bam*HI and screened by Southern blotting using a 299-bp *Not*I/*Bam*HI fragment as external probe. Targeted ES cell lines were aggregated with blastocysts from ICR mice (Charles River, Toronto, Canada) and implanted into pseudopregnant ICR females. The resultant chimeric males were mated with 129/Sv females. Heterozygous offspring were intercrossed into 129/Sv as well as ICR and C57BL/6 (Charles River) backgrounds to generate inbred and outbred DDR1-null mice. We did not observe any strain-dependent differences in the described phenotype of DDR1-null mice.

To generate the plasmid pRKDDR1-hAP, a cDNA fragment coding for the extracellular domain of DDR1 (amino acids 1 to 416) was fused in frame to the cDNA coding for human placenta alkaline phosphatase (hAP; a gift of A. Nagy).

Alkaline phosphatase staining. The plasmid pRKDDR1-hAP coding for the fusion protein between the extracellular domain of DDR1 and alkaline phosphatase was transiently expressed in human embryonic kidney fibroblast 293 cells (ATCC). Details of the transfection protocol have been published (33). The staining procedure with DDR1-hAP was modified from a published protocol (6). Transfected cells were resuspended in Hanks' balanced salt solution (HBSS) containing phenylmethylsulfonyl fluoride, aprotinin, and NaF as inhibitors. Cells were lysed by sonication. Aliquots of the lysate were incubated with 14-μm-thick cryosections from various mouse tissues for 90 min. Slides were washed five times with HBSS and fixed with a solution of 60% acetone, 3% formaldehyde, and 20 mM HEPES (pH 7.5) for 30 s. After incubation in HBSS buffer at 65°C for 20 min to inactivate endogenous alkaline phosphatase, slides were washed twice in AP buffer (100 mM Tris [pH 9.5], 100 mM NaCl, 5 mM $MgCl_2$). Samples were developed by incubation with AP buffer containing 0.17 mg of 5-bromo-4-chloro-3-indolyl phosphate/ml, 0.33 mg of nitroblue tetrazolium/ml, and 5 mM levamisole (all from Sigma) for 0.5 to 4 h. Slides were mounted with Aquamount (Paesel and Lorei, Hanau, Germany). Bacterial expression and purification of the extracellular domain of DDR has been described recently (35).

Histological and immunological staining. Sections (4 μm) of metatarsal bones from 2-week-old females were stained with hematoxylin-fast green-safranin O using standard procedures. To detect proliferating cells, 5-bromo-2′-deoxyuridine (BrdU; Sigma) and anti-BrdU monoclonal antibody (Boehringer Mannheim) were used as described previously (2). Apoptosis was analyzed by using the In Situ Cell Death Detection kit (Roche Diagnostics) according to the manufacturer's instructions.

For detection of DDR1, paraffin sections were incubated with an antibody directed against the C terminus of DDR1 (dilution, 1:100; Santa Cruz) and developed with aminoethylcarbazol staining. For Masson-Goldner staining, sections of paraffin-embedded mammary gland tissue were pretreated with hematoxylin for 10 min. After rinsing with water, sections were incubated with fuchsine-ponceau (0.2% [wt/vol] ponceau xylidine, 0.1% [wt/vol] acid fuchsine, 0.6% [vol/vol] acetic acid) for 6 min. Samples were rinsed with 1% acetic acid and treated with a solution of 3% (wt/vol) phosphomolybdic acid–3% orange G for 5 min. Sections were counterstained with 0.1% light green for 30 s.

Staining for the proliferation marker Ki-67 was essentially done as described previously (12). In brief, paraffin sections were dewaxed, oxidized with 1% H_2O_2 for 20 min, and equilibrated in Tris-buffered saline. The antigen was exposed by microwave treatment (800 W) for 2 min. Sections were incubated with a 1:50 dilution of rabbit anti-mouse Ki-67 antibody (Dianova, Hamburg, Germany) for 30 min. After washing with Tris-buffered saline, a peroxidase-coupled goat anti-rabbit secondary antibody was added for 30 min. Ki-67 expression was detected by 3,3′-diaminobenzidine tetrahydrochloride staining following a brief counterstain with hematoxylin.

Whole-mount mammary gland staining. The first abdominal mammary gland was removed, spread on a glass slide, and dried overnight. The gland was fixed and defatted in acetone for 24 h. Samples were stained with Harris' modified hematoxylin overnight and destained with several changes of 1% HCl in ethanol. The dye was fixed with a brief wash in 0.02% ammonium hydroxide. The tissue was cleared by incubation in xylene and mounted with Permount (BRL).

Western blot analysis. Embryonic or mammary gland tissue was homogenized in lysis buffer (33) using an Ultraturax blender (IKA-Werke, Staufen, Germany). One microgram of protein lysate was incubated with concanavalin A beads (Sigma) at 4°C for 3 h. Samples were washed three times with lysis buffer and analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis. Gels were transferred to nitrocellulose membranes and probed with a polyclonal antibody directed against DDR1 (Santa Cruz). For milk protein analysis, 5 μg of total lysate was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and blotted with a rabbit polyclonal serum raised against total mouse milk protein (kindly provided by N. Hynes). Blots were developed using enhanced chemiluminescence (Amersham).

Northern blot analysis. Mouse tissue was lysed in 4 M guanidinium hydrochloride. RNA was extracted by ultracentrifugation into a layer of 5.7 M CsCl. Twelve micrograms of total RNA was resolved with a 1% agarose gel in morpholinepropanesulfonic acid buffer and transferred to a Hybond N membrane (Amersham). Probes for β-casein (GTCTCTCTTGCAAGAGCAAGGGCC), α-lactalbumin (GGGCTTCTCACAACGCCACTGTTCA), WDNM1 (CAGAG CCCAGGCAGTAGTCATTGTC), and 28S rRNA (GAACAATGTAGGTAA GGGAAGTCGGCAAGCCGGATCCG) were ^{32}P end labeled with T4 polynucleotide kinase (27).

RESULTS

Visualization of DDR1 binding sites. The expression pattern of ligands for tyrosine kinase receptors can be studied using chimeric molecules containing the extracellular ligand-binding domain of the relevant receptor fused to alkaline phosphatase (6). We utilized this technique to generate a chimeric fusion protein, consisting of the human DDR1 ectodomain domain and human placenta alkaline phosphatase (called DDR1-hAP), in order to identify cells and tissues with the capacity to bind DDR1. Cell extracts from human embryonic kidney fibroblast 293 cells transfected with a plasmid coding for the DDR1-hAP fusion protein were used to probe cryosections of mice at different developmental stages. In a 6-day-old mouse, binding of DDR1-hAP was detected in several organs, particularly in the vertebrae, the skull and pubic bones, the urogenital tract, and the skin (Fig. 1A). At higher magnification, specific hybridization to dentin, alveolar bone, and stellate reticulum of a molar tooth was detected (Fig. 1B). In Fig. 1C and D, the whisker barrel and the periosteal collar of the clavicle are shown stained by the DDR1-hAP protein. In a control experiment, cell lysates lacking the DDR1-hAP fusion protein did not stain corresponding sections except in the lu-

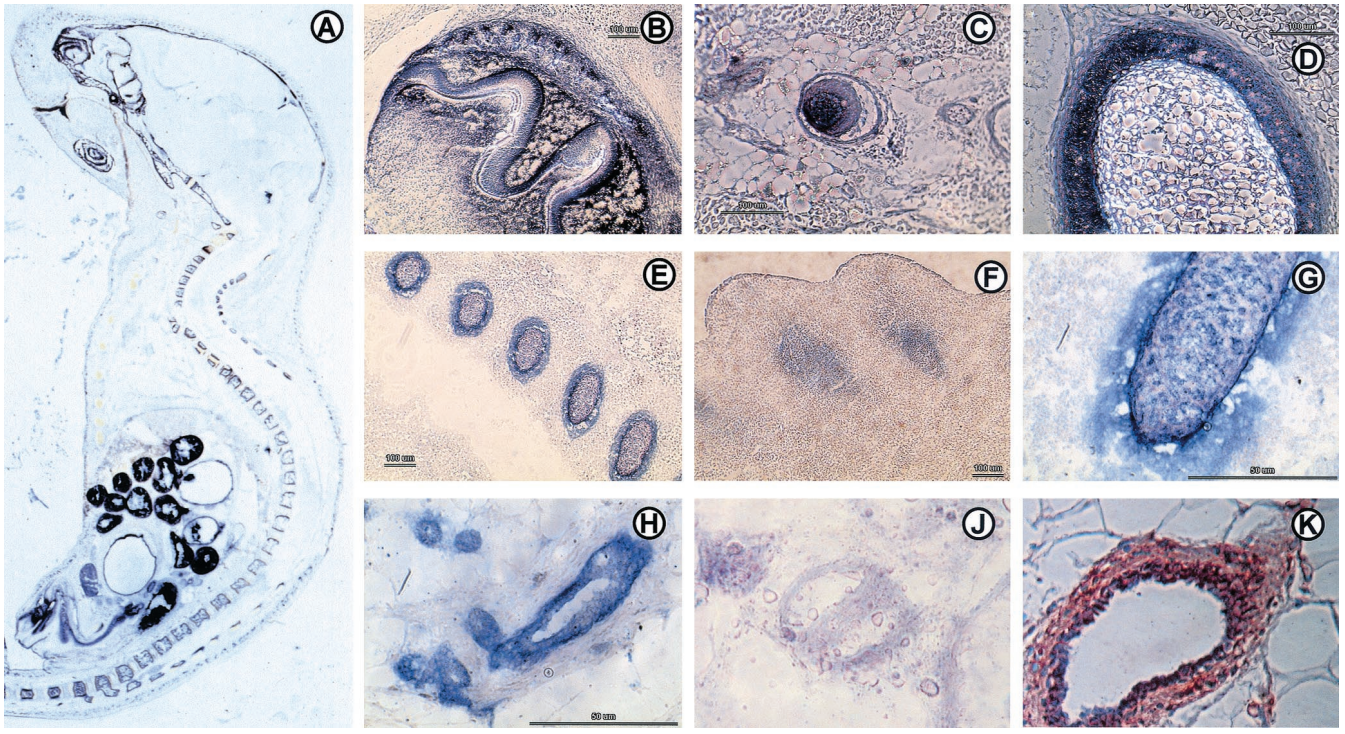

FIG. 1. Expression of DDR1-binding activity, shown by detection of DDR1-binding sites using a DDR1-hAP fusion protein. (A) Staining of a transverse section of a 6-day-old mouse with DDR1-hAP. DDR1-binding sites were detected in the skeleton, skin, and urogenital tract. (B to D) Staining of a molar tooth (B), the whisker barrel (C), and the periosteal collar of the clavicle (D). (E to G) Staining of a parasagittal section of an E13.5 mouse embryo with DDR1-hAP. Strong signals are seen in the ribs (E), the cartilage primordium of the metatarsal bones (F), and at higher magnification in the periosteum of the ribs (G). (H to K) Colocalization of DDR1-binding affinity and collagen expression in the mouse mammary gland. (H) Staining of mammary epithelial cells and adjacent myofibroblasts with DDR1-hAP using sections from a pregnant female at day 12.5 of gestation. (J) The binding of DDR1-hAP is competed with recombinant extracellular domain of DDR1. (K) Immunostaining for collagen type III in the mammary gland (brownish color).

men of the gut, possibly due to high endogenous alkaline phosphatase activity in the digestive tract (data not shown). The tissues labeled by the DDR1-hAP probe are considered to express DDR1 ligands, potentially specific collagens.

Tissue-specific binding of DDR1-hAP was also seen throughout mouse development. In parasagittal sections of an embryonic day 13.5 (E13.5) mouse embryo, strong DDR1 ligand activity was detected in the primordium of the ribs, vertebrae, and the cartilage primordium of the metatarsal bones (Fig. 1E and F). Particularly intense staining was seen in the periosteal collar of the developing ribs (Fig. 1G). Whole-mount staining of an E9.5 mouse embryo indicated expression of DDR ligand activity early in development (data not shown). In the mammary gland of pregnant mice at day 12.5 of gestation, DDR1-hAP bound to the epithelial cells forming ducts and alveoli and to the myofibroblasts in close proximity to the epithelium (Fig. 1H). Binding of DDR1-hAP to the mammary epithelium was efficiently competed by adding bacterially expressed extracellular domain of DDR1 to the staining reaction mixture (Fig. 1J). Immunohistochemical staining with an antibody against collagen type III coincided with the pattern of DDR1-hAP staining in the mammary gland (Fig. 1K).

Generation of DDR1-null mice. The prominent appearance of DDR1-binding activity during mouse embryogenesis and the dearth of information concerning DDR1 biological function prompted us to generate DDR1-null mice. Homologous recombination in ES cells was used to delete the first 12 exons of the DDR1 gene, including coding regions for the extracellular and transmembrane domains and part of the kinase domain (Fig. 2A). Correct integration of the neo^r cassette into the DDR1 locus was confirmed by Southern hybridization with 5′ and 3′ probes (data not shown). Blastocyst injection with the DDR1-targeted ES cell line resulted in several chimeric founder males that gave rise to $DDR1^{+/-}$ offspring. Heterozygous breeding produced wild-type, heterozygous, and mutant embryos in a Mendelian ratio (data not shown). We extracted proteins from embryos of one litter from heterozygous breeding and analyzed them by Western blotting with a DDR1-specific antibody. The absence of DDR1 protein expression in homozygous embryos indicated that the targeting event had generated a null mutation (Fig. 2B and C).

Homozygous mutant mice were born alive but remained smaller than their heterozygous littermates. On average, female DDR1-null mice had a 35% lower body weight than control animals (Fig. 3A). Mutant males remained smaller in the first few weeks after birth but gained additional weight after puberty. As a result, adult DDR1-null males were only about 10% smaller than wild-type mice. In both sexes, all organs were proportionally smaller. X-ray analysis revealed that the skeletons of 10-week-old DDR1-null mice appeared normal (Fig. 3B and C). Detailed analysis of several animals showed that the fibula bone was poorly calcified in the majority of the mutant animals (Fig. 3B and C, insert). Morphometric

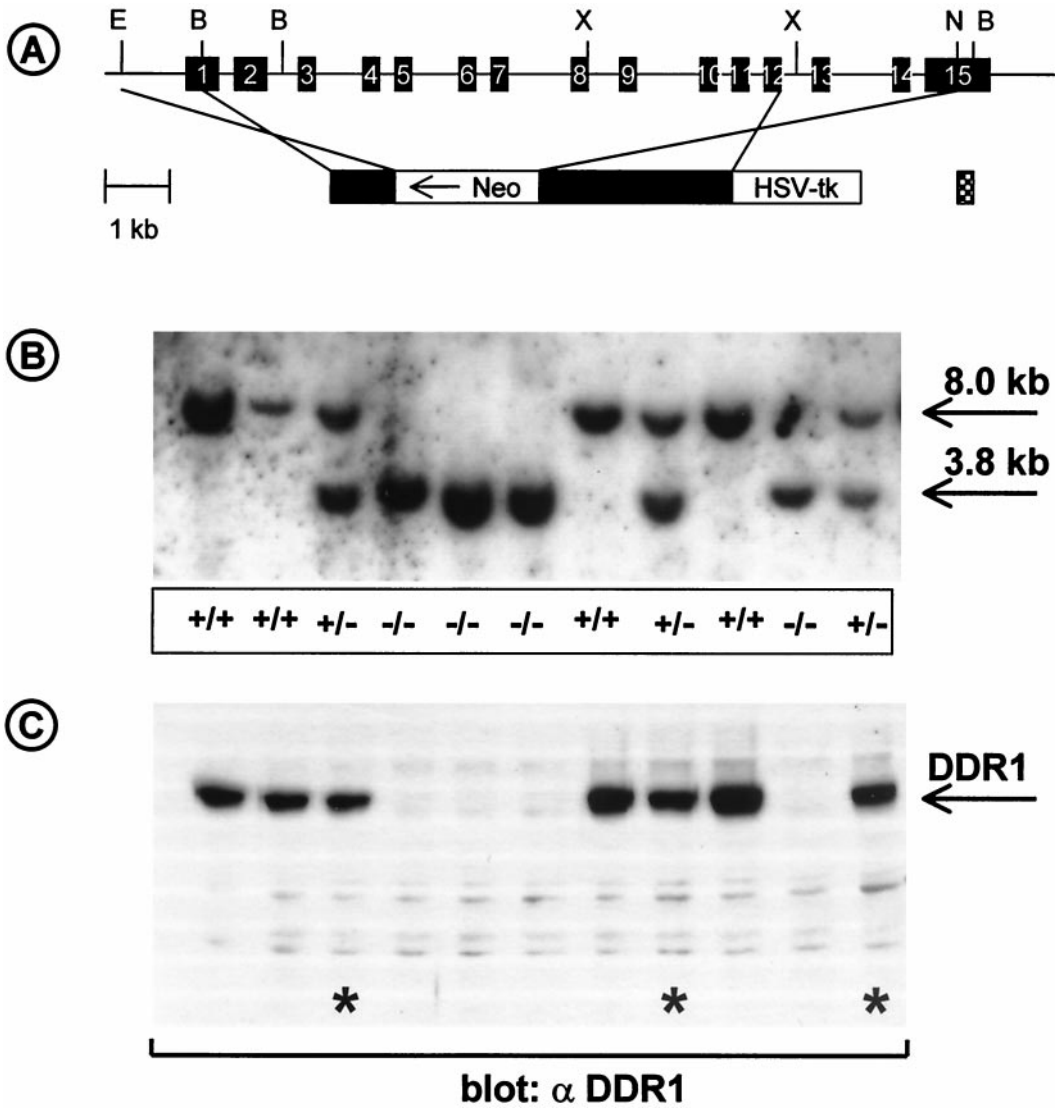

FIG. 2. Generation of DDR1-null mice. (A) Organization of the mouse DDR1 gene (top) and the targeting construct to generate DDR1-null mice (bottom). Exons are indicated by black boxes that are numbered beginning with the first coding exon as exon 1. The lengths of exons and introns are not to scale. Relevant restriction sites are indicated: E, *Eco*RI; B, *Bam*HI; X, *Xho*I; N, *Not*I. The checkered box represents the external probe used for genotyping of ES cells and mice. (B) Southern blot analysis of DNA isolated from the embryos of a heterozygous intercross (+/+, wild-type; +/−, heterozygous; −/−, homozygous). An 8.0-kb *Bam*HI fragment corresponds to the wild-type allele, and a 3.8-kb fragment corresponds to the mutant allele. (C) Western blot analysis of lysates generated from the same embryos as in panel B. DDR1 is detected as an approximately 130-kDa protein. Asterisks denote heterozygous embryos with reduced DDR1 expression.

measurement indicated that mutant animals presented a narrower pelvis than control animals (data not shown).

To investigate the reason for the dwarfism in DDR1-null mice, we examined the growth plates by staining sections of metatarsal bones with hematoxylin-fast green-safranin O. As shown in Fig. 3D and E, the length of the zone of hypertrophic cartilage in 2-week-old DDR1-null mice appeared unaltered from that in the control. To measure chondrocyte proliferation, mice were injected for 90 min with BrdU. BrdU-positive cells were counted in sections of the tibia from 2-, 7-, and 16-week-old animals (Fig. 3F and G and data not shown). Quantitative analysis revealed no significant difference in the number of BrdU-positive cells in control (Fig. 3F) versus mutant (Fig. 3G) animals. Since reduced bone growth also could be due to increased chondrocyte apoptosis, we performed a terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick end labeling (TUNEL) assay on sections of the tibia from 4- and 8-week-old animals. As seen in Fig. 3H and J, no difference in the rate of chondrocyte apoptosis could be detected between control and knockout tissues.

A high percentage of DDR1-null animals was not able to control their ear movement. One or sometimes both ears were curled back towards the body (Fig. 4A). DDR1-hAP staining showed high expression of DDR1 ligand proteins in the elastic ear cartilage, suggesting that DDR1 is necessary for proper formation of the mouse ear (Fig. 4B).

Implantation and lactation are affected in DDR1-null mice. Mating between DDR1-null females and either mutant or con-

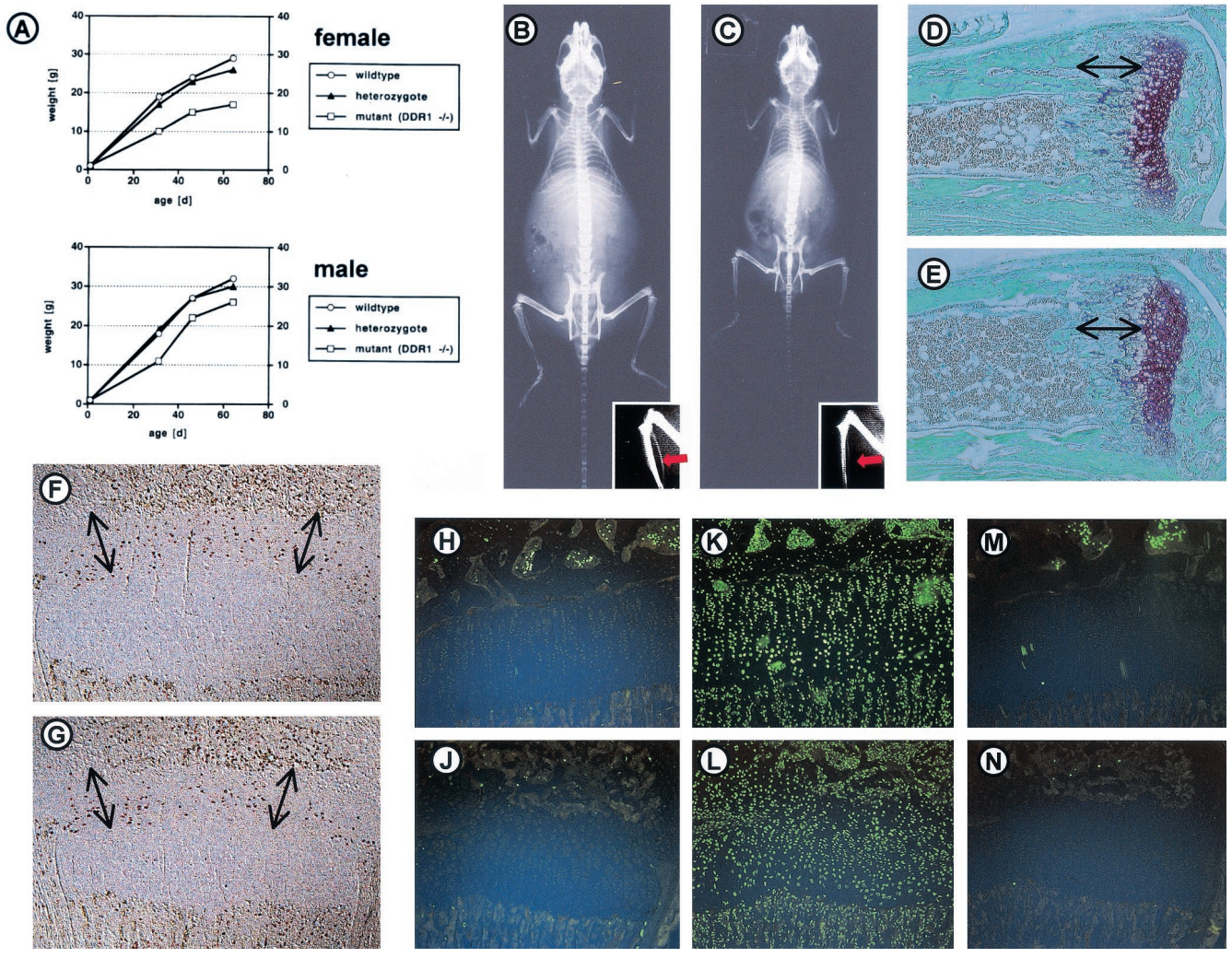

FIG. 3. Dwarfism of DDR1-null mice. (A) Growth curves of male and female offspring during the first 10 weeks of age. One set of growth curves, which is representative of six measurements taken from different litters during the same time period, is depicted. (B and C) X-ray analysis of heterozygous (B) and mutant (C) 10-week-old females. The lack of proper mineralization of the fibula bone is shown by an arrow in the insert. (D and E) Hematoxylin-fast green-safranin O staining of growth plates in heterozygous (D) and mutant (E) metatarsal bones of 2-week-old mice. The comparable length of the growth plate in the mutant and control section is indicated by an arrow. (F and G) BrdU-stained sections of tibias from 2-week-old heterozygous (F) and mutant (G) mice. Arrows mark the length of the proliferative zone. (H to N) TUNEL assay with sections from the tibia of 4-week-old wild-type (H, K, M) and mutant (J, L, N) animals. No increase in chondrocyte apoptosis was seen in DDR1-null chondrocytes (H and J). Sections were pretreated with DNase I as positive control (K and L) or incubated without enzyme as negative control (M and N).

trol males was often unsuccessful. The appearance of vaginal plugs suggested that mating could take place. On dissection of mutant females at day 3.5 of gestation, we observed a normal number of mature preimplantation blastocysts in the uterus (data not shown). No decidual swelling was observed in mice dissected after day 4.5 of gestation. Blastocyst transfer into pseudopregnant wild-type mothers resulted in a normal litter. We therefore reasoned that the lack of implantation might be due to a maternal defect. To pursue this notion, we probed uterine sections from wild-type mice at days 2.5 and 4.5 of gestation with the DDR1-hAP fusion protein (Fig. 4C and E). The longitudinal muscles and the myometrium of the preimplantation uterus (E2.5) expressed high levels of DDR1-binding sites (Fig. 4C). In contrast, the uterine stroma showed a much lower intensity of staining. Using immunohistochemistry to detect DDR1, we found a striking correlation between receptor and ligand (presumably collagen) expression (Fig. 4D). The longitudinal muscles and the outer layer of the myometrium displayed expression of DDR1, whereas it was absent in the glandular epithelium and in the stroma. After implantation at E4.5, cells in the decidual zone around the embryo stained strongly with the DDR1-hAP reagent (Fig. 4E). The expression of DDR1 colocalized to the decidual cell population (Fig. 4F). In contrast, the embryo proper was negative for ligand as well as for receptor expression. The concomitant expression of receptor and ligand suggests that DDR1 is necessary for the peri-implantational adhesion between the luminal epithelium of the uterus and the blastocyst.

In approximately 20% of DDR1-null females, implantation took place and the mice gave birth to a full litter. One day after

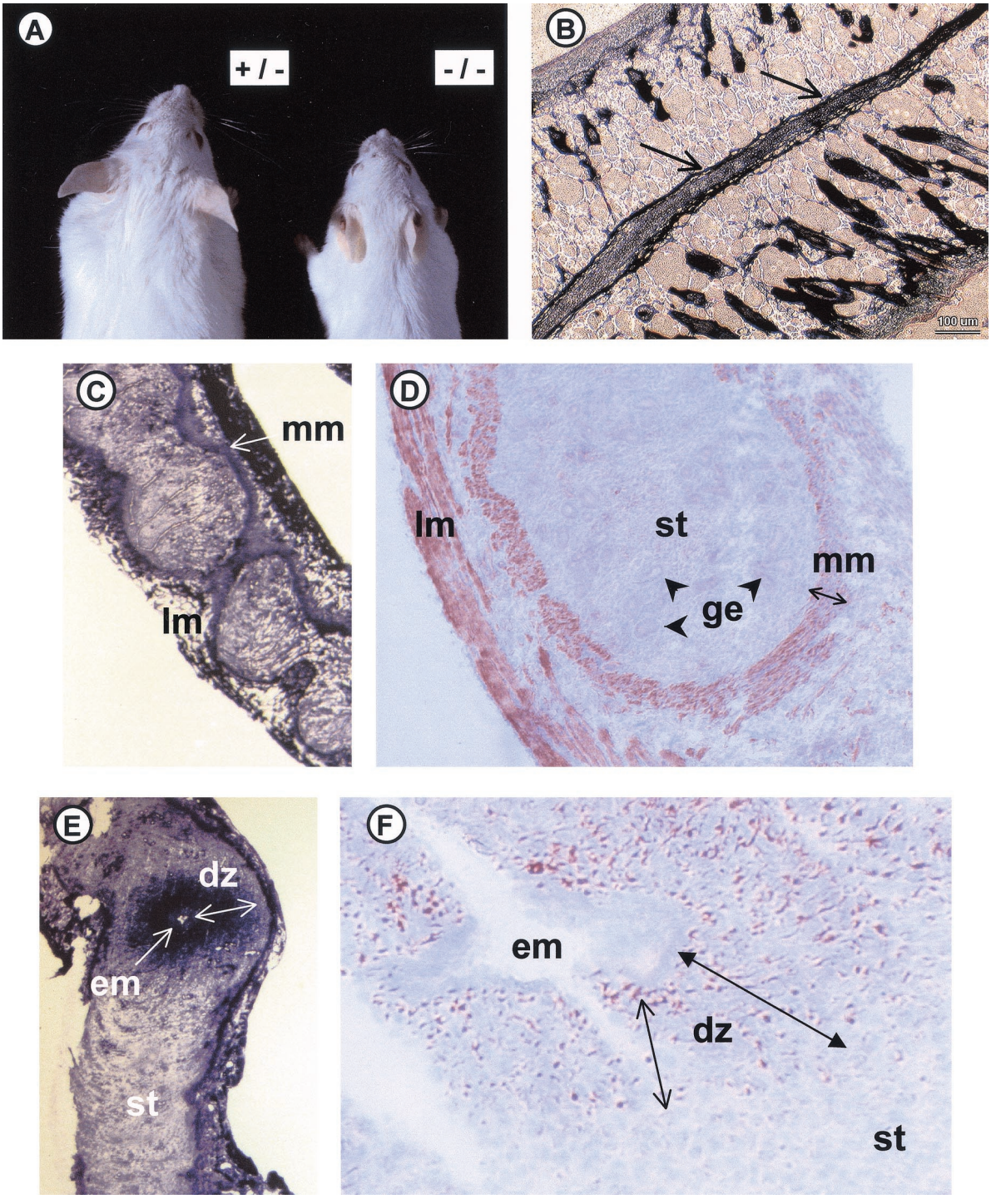

FIG. 4. Defects of DDR1-null mice in ear and placental development. (A) Phenotypic appearance of ears in control and mutant mice. Mice were 3 months of age and were anesthetized prior to photography. (B) Detection of DDR1-binding affinity in the ear cartilage using the DDR1-hAP fusion protein (indicated by arrows). The hair follicles are stained as well. (C to F) DDR1-hAP staining (C and E) and DDR1 immunostaining (D and F) in pre- (E2.5; C and D) and post- (E4.5; E and F) implantational uteri in wild-type mice. Abbreviations: mm, myometrium; lm, longitudinal muscle; st, stroma; gl, glandular epithelium; dz, desidual zone; em, embryo proper.

birth, all of the pups appeared malnourished. Only small amounts of milk were detected in their stomachs. When left with their mother, all pups died within the following days. The pups could be saved by transferring them to a wild-type foster mother shortly after birth. To investigate the defect in lactation seen in DDR1-null mice, we analyzed the mammary glands of mutant females during pregnancy. Sections of mammary tissue from mutant mice at day 18.5 of gestation revealed a much

more condensed alveolar structure (Fig. 5B) compared to the control (Fig. 5A). The heterozygous control showed a large number of lipid vesicles in the mammary epithelium, whereas DDR1-null mice appeared to have few lipid vesicles. Whole-mount analysis of the E18.5 mammary gland confirmed that the fat pad of mutant mice was almost entirely filled with ducts (Fig. 5D and F), which had a more distended appearance than in controls (Fig. 5C and E).

To study lactation, sections of mammary tissue from mutant and control animals 1 day postpartum were taken. In control females, the adipose tissue had disappeared and was entirely replaced by alveolar structures filled with milk (Fig. 5G). In contrast, the mutant mammary gland tissue was largely composed of adipocytes (Fig. 5H). The alveoli were predominantly condensed, and very little milk had been secreted. At day 2 after birth, the alveoli in the mutants started to collapse and the previously formed epithelium began to regress (Fig. 5J and K). We suspected that the lack of appropriate mammary gland differentiation in DDR1-null mice might be caused by premature apoptosis during pregnancy. To test this hypothesis, we analyzed sections of mutant mammary gland tissue by TUNEL staining, but we could not see any increase in DNA fragmentation above that in wild-type controls (data not shown).

To gain further understanding of the developmental defect of the mammary gland in DDR1-null mice, we analyzed early ductal growth in the mammary fat pad of 3-week-old littermates. In heterozygous animals, the ducts, which originate from the nipple, reached into the center of the mammary gland, thereby passing the lymph node (Fig. 6A). In mutant animals, ductal growth was considerably delayed. At 3 weeks of age, only the first quarter of the gland was filled with ducts (Fig. 6B). Notably, the terminal end buds in the mutant mice appeared to be enlarged compared to those in control mice. At 3 months of age, the fat pads of both wild-type (Fig. 6C) and mutant (Fig. 6D) animals were filled with epithelial ducts. In sections from mutant mice, we detected a substantial increase in the number and diameter of the ducts (Fig. 6C and D). To further characterize the abnormal development, we stained the extracellular matrix in breast sections of 3-month-old female mice using the method of Masson-Goldner. We detected a substantial increase in extracellular matrix deposition in the mutant mammary gland above that in the wild type (Fig. 6C and D). In the mutant tissue, collagenous extracellular matrix was not only found along the epithelial ducts but also was widespread in the adipose tissue (Fig. 6C and D, inserts). To quantify the proliferation rate in the mutant epithelium, we analyzed sections with an antibody to Ki-67, which has been found to label all cells that are not in the G_0 phase of the cell cycle (12). The mutant epithelium had approximately four to five times more Ki-67-positive cells (Fig. 6F) than the heterozygous control (Fig. 6E). Taken together, the invasion of the mammary fat pad by epithelial cells during puberty is delayed in DDR1-null mice, but the epithelium itself is hyperproliferative, resulting in an increased number of enlarged ducts.

To further support the notion that DDR1 is essential for mammary gland differentiation during pregnancy and lactation, we analyzed DDR1 expression by immunohistochemistry and Western blotting. As shown in Fig. 6G, luminal epithelial cells of a wild-type mammary gland express increased amounts of DDR1 in comparison to the underlying smooth muscle cells and adipocytes. Western blot analysis showed an increase of DDR1 expression during pregnancy, with highest levels at late pregnancy (Fig. 6H). The expression persisted during lactation but dramatically decreased at the time of involution.

Gene expression in the DDR1-null mammary gland. To gain a more detailed understanding of why DDR1-null mice have insufficient milk production, we tested the expression of milk proteins in the mammary gland of mice at 2 days postpartum. We found reduced expression of a 26-kDa milk protein and almost complete absence of an 82-kDa protein in lysates from DDR1-null mammary gland (Fig. 7A). The mRNA expression of individual milk proteins was quantified using Northern blotting. We found that DDR1 mutant mice have mRNA for the milk proteins β-casein, α-lactalbumin, whey acidic protein (WAp), and the milk protein transcription regulator WDNM1 in amounts comparable to control animals during lactation (Fig. 7B and C). Hybridization with a probe against 28S rRNA indicated that the reduction of β-casein and WDNM1 mRNA at late pregnancy (day 18.5 of embryonic development, mutant) and mid-lactation (day 5 postpartum, wild type) appeared to be due to differences in sample loading.

DISCUSSION

We have investigated the expression of potential DDR1 RTK ligands using a chimeric receptor fusion protein. This approach has identified the growing skeleton, the skin, the kidney, and the urogenital tract as major sites of DDR1 receptor binding. All these tissues abundantly express fibrillar as well as basement membrane collagens, consistent with the in vitro binding and activation of DDR1 by type I to type V collagen (33). In the mammary gland, we demonstrated colocalization of DDR1 binding sites with the expression of collagen type III. However, these results do not exclude the possibility that other ligands are involved in DDR1 recognition. The sites with highest receptor and ligand expressions were the developing teeth, the skin, and the vibrissae. Surprisingly, we did not detect DDR1-hAP binding in neuronal tissues, although DDR1 is expressed in the developing and adult mouse brain, in particular in the cortex, hippocampus, and cerebellum (5, 29).

Ablation of DDR1 in the mouse resulted in a severe postnatal growth reduction. Despite strong binding of the DDR1-hAP fusion protein to the skeleton, DDR1 appears to have no general influence on bone formation or mineralization. Furthermore, chondrocyte proliferation, apoptosis, and the morphology of the growth plates in the tibia and metatarsus are normal. It is possible that a hormonal stimulus that regulates bone and tissue growth may be reduced in DDR1-deficient mice. The apparent lack of any embryonic phenotype in DDR1 mutant mice is surprising, since DDR1 is highly expressed throughout embryogenesis (29). It is possible that DDR1 and DDR2 receptors have overlapping functions during fetal development (16).

In the mouse, the process of implantation begins with the contact between trophoectodermal cells of the embryo and the luminal epithelium of the uterus. The initial attachment of the blastocyst induces local apoptosis of the epithelium, followed by degradation of the underlying basement membrane. During decidualization, trophoectodermal cells invade the uterine stroma and form the implantation chamber (26). These pro-

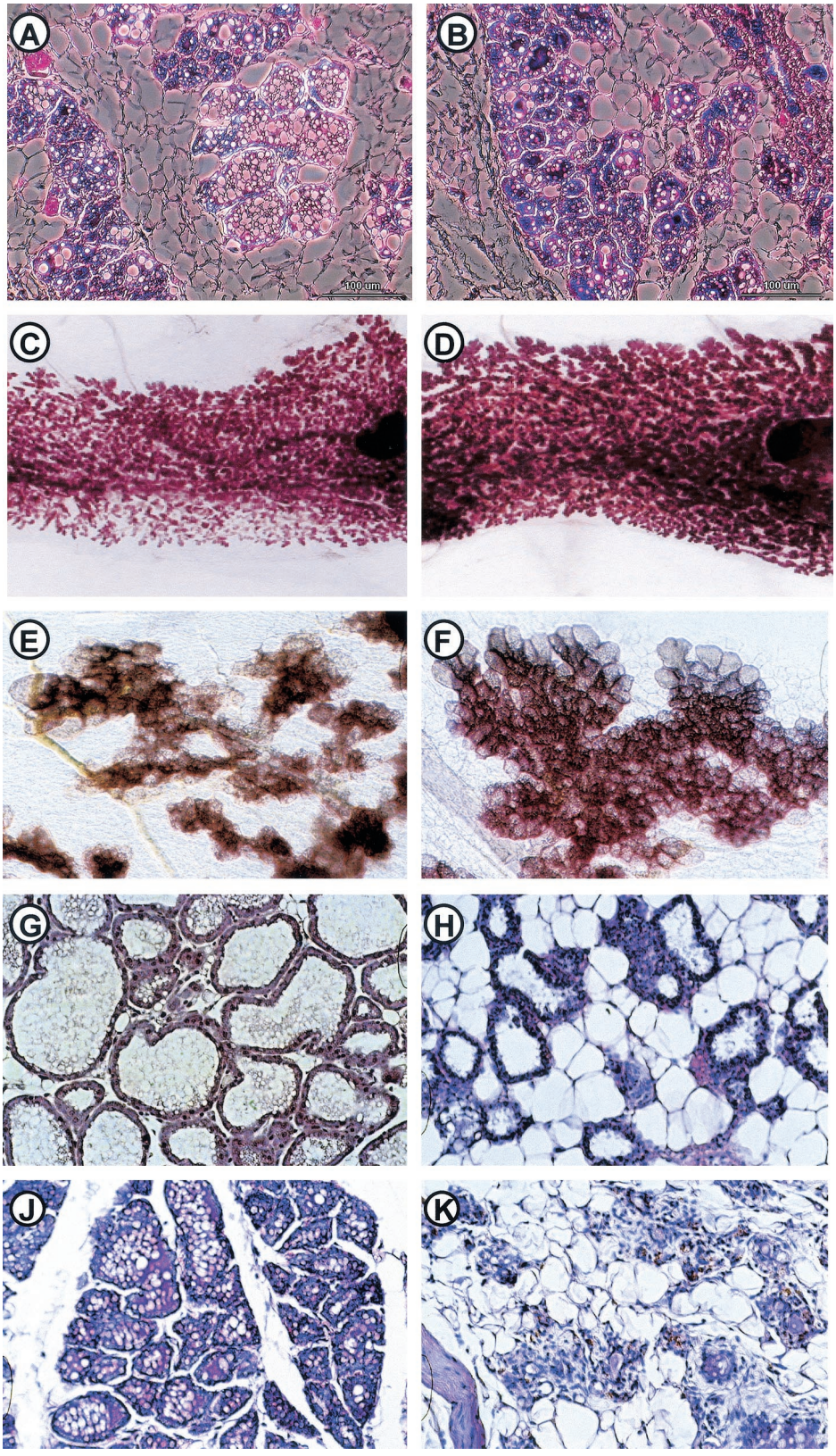

FIG. 5. Mammary gland defects in DDR1-null mice. (A and B) Hematoxylin-eosin stain of late pregnancy (day 18.5 of gestation) mammary glands of heterozygous (A) and mutant (B) mice. (C to F) Whole-mount analysis of control (C and E) and mutant (D and F) mammary glands of day 18.5 pregnant females. Pictures are taken at low (C and D) and higher (E and F) magnification. (G and H) Mammary gland morphology of heterozygous (G) and mutant (H) mice 1 day postpartum. Note the almost complete absence of milk secretion into the lumens in panel H. (J and K) Regressed mammary epithelium in mutant mice (K) compared to that in control mice (J) 2 days postpartum.

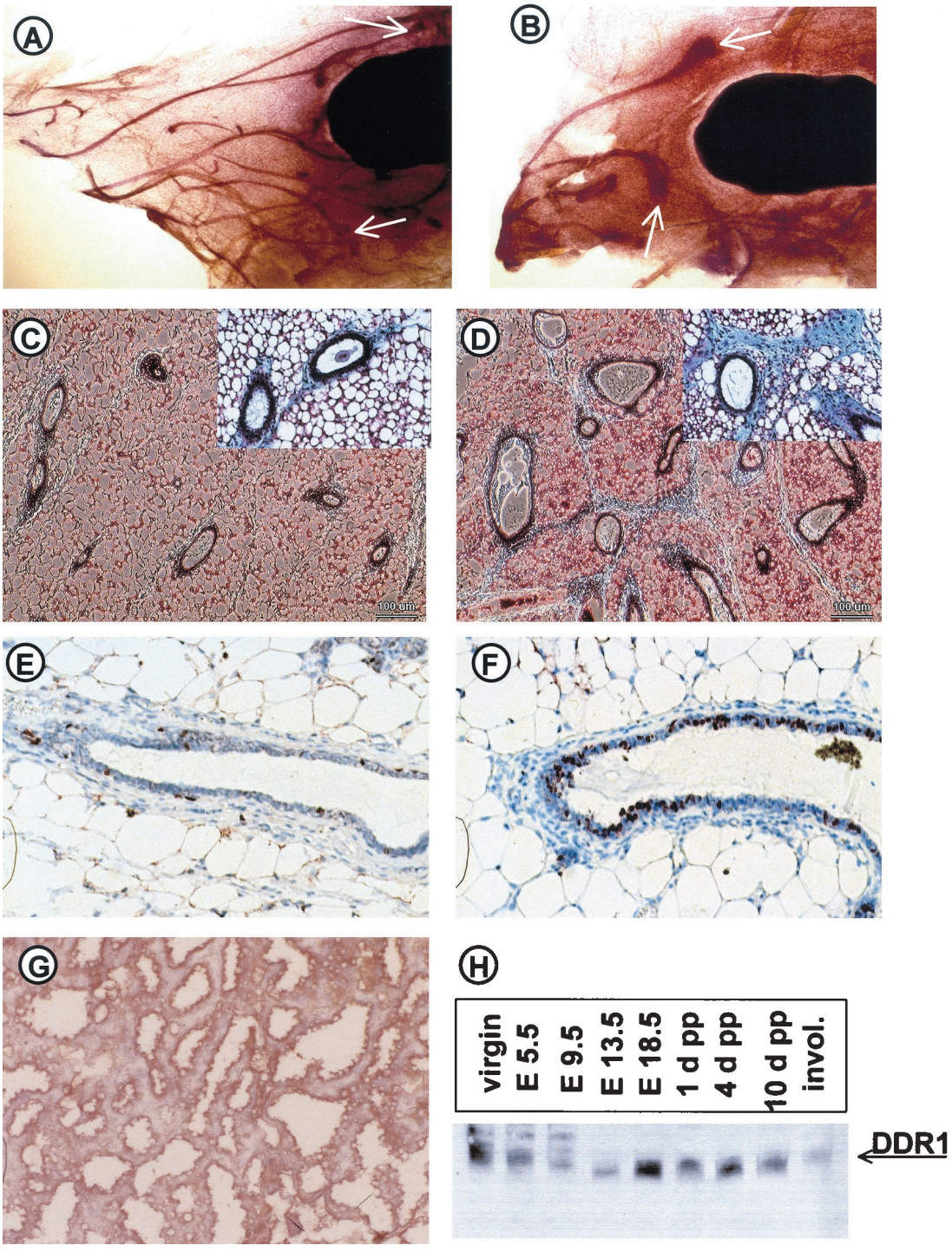

FIG. 6. Mammary gland development during puberty and pregnancy in DDR1-null mice. (A and B) Whole-mount analysis of the developing mammary gland in 3-week-old heterozygous (A) and mutant (B) mice. Some terminal end buds are marked by arrows. (C and D) Masson-Goldner stain of virgin heterozygous (C) and mutant (D) mammary glands. Note the enlarged ducts and the increase in extracellular matrix deposition in the adipose tissue (insert). (E and F) Ki-67 staining of virgin control (E) and mutant (F) mammary gland. The number of Ki-67-positive (red-labeled) nuclei is approximately five times higher in the mutant tissue. (G) Immunolocalization of DDR1 in a lactating mammary gland. Specific staining (brownish color) is seen in the luminal epithelial cells. (H) Western blot analysis of DDR1 expression during pregnancy and lactation.

esses are accompanied by a drastic remodeling of the extracellular matrix, involving proteases such as gelatinases A and B, also called MMP2 and MMP9 (8). DDR1 is expressed in the decidua during that time of implantation (Fig. 4F), as are the ligands for DDR1 including the α1 and α2 chain of type VI collagen, which are specifically expressed in the uterine epithelium around the implantation site (10). Therefore, we propose a model in which DDR1 expressed in the decidual cells is

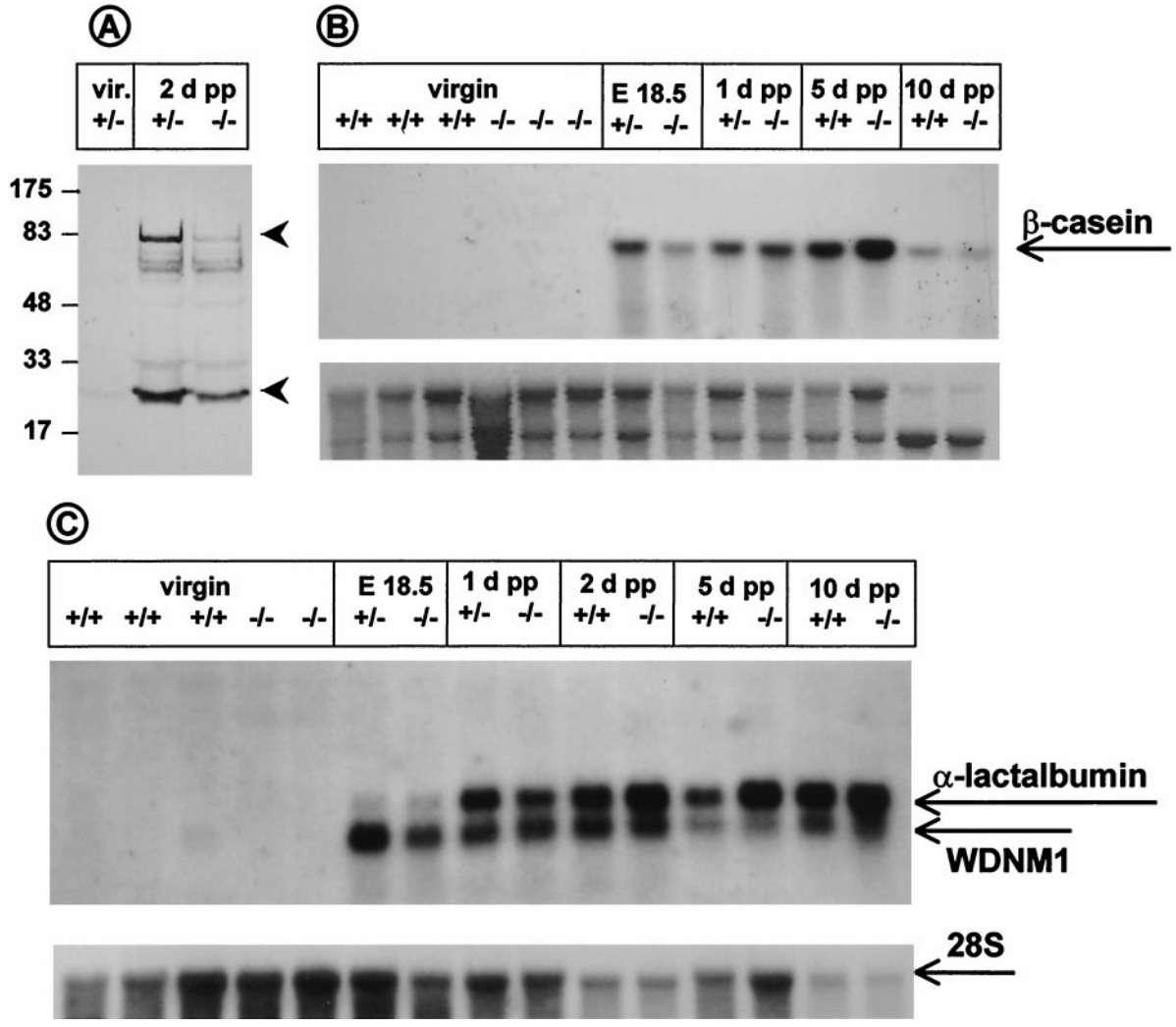

FIG. 7. Expression of milk proteins in DDR1-null females. (A) Western blot analysis of milk protein expression in lysates from mouse mammary glands 2 days postpartum. Arrows indicate milk proteins reduced or absent in DDR1-null animals. Northern blot analysis of β-casein (B) and α-lactalbumin together with WDNM1 (C) expression during mammary gland development of mutant and control mice. The lower strip in panel B shows the ethidium bromide-stained gel, and the lower strip in panel C shows reanalysis of the blot with a 28S rRNA probe.

necessary for MMP production and/or reorganization of collagenous matrix.

In female mice lacking DDR1, development of the mammary gland is delayed and deviates rather early from the normal differentiation path. Mutant mice show enlarged terminal end buds due to aberrant ductal growth. The number of secondary ducts is increased, the ducts have wider lumens, and significantly larger amounts of extracellular matrix are deposited around the ducts. In the absence of DDR1, the mammary epithelium of virgin mice is hyperproliferative. During pregnancy, the lack of DDR1 perturbs the lobuloalveolar proliferation and differentiation, resulting in a large number of alveoli that are not able to secrete milk. The fact that we detected normal levels of milk protein transcripts in mutant mammary gland suggests that protein translation is perturbed in the mutant tissue.

During pubertal development, the mammary gland is one of the most active organs undergoing cell growth and differentiation. In a newborn female mouse, the mammary gland only consists of a fat pad that is attached to the nipple. From the nipple, a primary duct opens into the adipose tissue. Around the third postnatal week, the terminal end bud at the tip of the primary duct branches and invades the fat pad. This sequence of events is altered in DDR1-deficient mice. While mammary duct outgrowth is delayed, the primary ducts and the terminal end buds are enlarged compared to those in control mice. A severalfold-higher proliferative rate of mutant cells resulted in the formation of ducts with a wider lumen. In addition, the mammary epithelium in mutant mice is lined by a substantially increased amount of collagenous extracellular matrix. During pregnancy, accelerated ductal growth and aberrant lobuloalveolar differentiation continue in the mutants. At birth, the alveoli show intracellular lipid production and deposition but fail to secrete milk into the central lumen. As a result, DDR1-null mice are unable to produce sufficient milk, despite normal transcriptional activation of milk proteins. At the time of lac-

tation, when we detected the highest protein expression of DDR1 (Fig. 6H), the most dramatic phenotype in DDR1-null mice is apparent.

The results presented here suggest that DDR1 has at least two control functions in the mammary gland: (i) epithelial proliferation and (ii) synthesis of collagenous extracellular matrix. Since both processes were up-regulated in the mutant mice, a general negative regulatory function can be attributed to DDR1. The hyperproliferation in the mutant mammary gland suggests that one potential physiological function of DDR1 is to suppress cell proliferation. The aberrant deposition of extracellular matrix suggests that another function of DDR1 is to program cellular differentiation and to mediate cell-matrix contacts. Using C2C12 myofibroblasts overexpressing dominant-negative DDR1, we recently showed that cellular differentiation can be efficiently blocked by inhibition of DDR1 signaling (35).

A central regulator in cell proliferation is the Ras/mitogen-activated protein kinase pathway, which is activated by the Shc-Grb2-Sos complex. After tyrosine kinase receptor stimulation, Shc binds to the NPXpY motifs, for example, in activated epidermal growth factor or Trk receptors, becomes phosphorylated, and thus allows the Grb2-Sos complex to bind (21). In human mammary carcinoma T-47D cells, the LLXNPXpY sequence in the b-isoform of DDR1 binds to Shc. In contrast to activation of epidermal growth factor receptor or Trk, collagen-induced activation of DDR1 does not result in tyrosine phosphorylation of Shc (35). In the absence of DDR1, more Shc molecules are potentially able to interact with growth-promoting receptors, which results in an enhancement of cell proliferation. It is therefore tempting to speculate that DDR1 suppresses mitogen-activated protein kinase activation by recruiting Shc into a nonactivating complex. However, other mechanisms can be postulated: DDR1 could influence proliferation as a potential upstream regulator of cell cycle control proteins, for example, p53 (28). Inhibition of cell cycle progression has been recently shown in smooth muscle cells stimulated with fibrillar collagen and in lung epithelial cells stimulated with collagen type V, suggesting that some of these processes may be mediated by DDR1 (15, 20)

Malformation of the mammary gland and lactational failure have been previously described in several other mouse mutants. For example, lobuloalveolar development is impaired in mice lacking the prolactin receptor and its targets, Stat5a and Stat5b (31). Prolactin signaling regulates the expression of milk proteins such as WAP. Whereas in Stat5a-deficient mice WAP gene expression is absent, we could detect transcripts for WAP and β-casein in DDR1-null mice, suggesting that milk protein expression is not regulated by DDR1 (18). This is supported by the finding that Stat5 expression or tyrosine phosphorylation was not significantly altered in DDR1-null animals (data not shown).

Similar to the DDR1-null mice, the majority of mice with a deletion in the colony-stimulating factor 1 (CSF-1) gene lack proper blastocyst implantation (25). Pregnant CSF-1-null mice show premature lobuloalveolar outgrowth with an excess of branch density. As with DDR1-null mice, postpartum alveoli of CSF-1-deficient mice are widely dispersed in the fat pad and lack a luminal opening. Recent evidence suggests that the defects in CSF-1-null mice are primarily caused by ovulation problems, whereas DDR1-null mice ovulate normally (reference 7 and data not shown). It is tempting to speculate that CSF-1R and DDR1 RTKs complement each other during mammary gland development. Whereas the CSF-1R is triggered by exocrine stimuli, in particular by CSF-1 secreted from the uterus, DDR1 may be activated in the mammary epithelium by directly contacting the collagenous matrix of connective tissue.

The results presented here identify DDR1 as an essential gene product in mammary gland development and suggest a role for DDR1 in human breast carcinoma progression.

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