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Maternal Smad3 Deficiency Compromises Decidualization in Mice

Kun-Qing Zhao,[1,2] Hai-Yan Lin,[1*] Cheng Zhu,[1] Xiao Yang,[3,4] and Hongmei Wang[1*]

[1] *State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, Beijing 100101, China*

[2] *Graduate School of Chinese Academy of Sciences, Beijing 100039, China*

[3] *State Key Laboratory of Proteomics, Genetic Laboratory of Development and Diseases, Institute of Biotechnology, Beijing 100071, China*

[4] *Model Organism Division, E-institutes of Shanghai Universities, Shanghai Jiaotong University, Shanghai 200025, China*

ABSTRACT

Transforming growth factor (TGF)-β and activin, members of TGF-β superfamily, are abundantly expressed in the endometrium and regulate decidualization of endometrial stroma. Smad2 and Smad3 are receptor-regulated Smads (R-Smads) that transduce extracellular TGF-β/activin/Nodal signaling. In situ hybridization results showed that Smad3 was highly expressed in the decidual zone during the peri-implantation period in mice. By using artificial decidualization, we found that Smad3 null mice showed partially compromised decidualization. We therefore hypothesized that Smad2 might compensate for the function of Smad3 during the process of decidualization. Smad2 was also highly expressed in the decidual zone and phosphorylated Smad2 was much more abundantly increased in the deciduoma of Smad3 null mice than for wild-type (WT) mice. We further employed an in vitro uterine stromal cell decidualization model, and found that decidual prolactin-related protein (dPRP) and cyclin D3, which are well-known markers for decidual cells, were significantly down-regulated in Smad3 null decidual cells, and were much more significantly reduced when the expression of Smad2 was simultaneously silenced by its siRNA ($P < 0.05$). However, the expression levels of dPRP and cyclin D3 remained the same when Smad2 was silenced in WT decidual cells. Collectively, these findings provide evidence for an important role of Smad3 in decidualization and suggest that Smad2 and Smad3 may have redundant roles in decidualization. J. Cell. Biochem. 113: 3266–3275, 2012.

KEY WORDS: DECIDUALIZATION; Smad3; Smad2; TGF-β

Smads are pivotal intracellular transcription factors that mediate transforming growth factor (TGF)-β signals. TGF-β signaling pathway regulates a multitude of cellular processes such as cell proliferation, differentiation, migration, and apoptosis during embryogenesis and in mature tissues [Massague, 2000]. A TGF-β ligand elicits its cellular response by binding to and activating membrane receptors with serine/threonine kinase domains in their cytoplasmic region. The activated receptors then phosphorylate the receptor-regulated Smads (R-Smad). Phosphorylated R-Smads subsequently form a complex with Co-Smad, Smad4, and the activated Smad complex cumulates in the nucleus and regulates the transcription of target genes with the aid of other cofactors. The subfamily of R-Smads contains five members: Smad2 and Smad3, which respond to signaling by the TGF-β/activin/Nodal subfamily, and Smad1, Smad5, and Smad8, which mediate the signaling by bone morphogenetic protein (BMP)/growth differentiation factor (GDF)/Müllerian inhibiting factor (MIS) subfamily [Derynck et al., 1998; Shi and Massague, 2003].

Successful implantation is a crucial event in mammalian reproduction. Embryo implantation requires the interactions

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*Correspondence to: Dr. Hongmei Wang or Dr. Hai-Yan Lin, State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences, 1 Beichen West Road, Chaoyang District, Beijing 100101, China.
E-mail: wanghm@ioz.ac.cn; linhy@ioz.ac.cn

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between the blastocyst and the receptive uterus. The process of implantation is divided into three serial stages: apposition, adhesion, and penetration [Enders and Schlafke, 1969]. With the initiation of blastocyst attachment, uterine stromal cells surrounding the blastocyst undergo decidualization, which initiates at the antimesometrial site where the blastocyst implants, and then spreads to the mesometrial endometrium [Abrahamsohn and Zorn, 1993]. In the mouse, stromal cells surrounding the embryo immediately cease proliferating and form the primary decidual zone (PDZ) [Paria et al., 1999]. One of the most important physiological features of the PDZ is avascular [Abrahamsohn and Zorn, 1993]. The PDZ serves as a barrier to protect the embryo exposure from immunocompetent cells [Wang et al., 2004]. Stromal cells adjacent to the PDZ continue to proliferate and differentiate, and form the secondary decidual zone (SDZ), which is well established on day 7 of pregnancy [Dey et al., 2004]. Artificial decidualization can be induced by intraluminal oil infusion in the receptive pseudopregnant uterus, or uterus that has been appropriately prepared by ovarian steroids [Kennedy, 1983; Lim et al., 1997].

TGF-β and activin play important roles during the process of decidualization. TGF-β1 and TGF-β2 are expressed in human endometrial stromal, epithelium, and decidual cells [Gold et al., 1994; Godkin and Dore, 1998; Simpson et al., 2002], and their mRNAs are increased during the decidualization of human endometrial stromal cells in vitro [Popovici et al., 2000; Tierney et al., 2003]. Furthermore, TGF-β1 promotes the decidualization of isolated human endometrial stromal cells in vitro [Kim et al., 2005; Chang et al., 2008]. However, Kubota et al. [1997] reported that TGF-β1 attenuated the release of prolactin (PRL), a well-established marker of decidualization, in decidual cells from early pregnancy of human in vitro. Activins are expressed during the proliferative and secretory phases and the expression of activins is dramatically up-regulated during the process of decidualization in the human [Otani et al., 1998; Jones et al., 2000]. Activins can promote the decidualization [Tierney and Giudice, 2004], partly due to stimulation of matrix metalloproteinases (MMPs) in endometrial cells [Jones et al., 2006]. However, despite the fact that TGF-βs and activins are important molecules involved in decidualization, the mechanism whereby TGF-βs and activins exert their effect on decidualization remains to be elucidated. In this study, we therefore employed knockout mice of Smad3, which is a pivotal intracellular mediator of TGF-β subfamily, to study the role of Smad3 in decidualization, and we found that during this process Smad3 was an essential factor and Smad2 and Smad3 functioned redundantly.

MATERIALS AND METHODS

ANIMALS

Smad3 knockout mice were generated as previously described [Yang et al., 1999]. Breeding pairs of Smad3 heterozygous mice were kindly provided by Professor Xiao Yang (Institute of Biotechnology, AMMS, Beijing, China) and were maintained on the same background in our animal care facility. Heterozygous females ($Smad3^{+/-}$) were mated with heterozygous males ($Smad3^{+/-}$) to generate WT, $Smad3^{+/-}$ and homozygous ($Smad3^{-/-}$) offsprings. The genotypes of offsprings were determined by PCR analysis of tail DNA as previously described [Yang et al., 1999].

The CD1 mice used in this study were purchased from Vital River Lab Technology CO., Ltd. (Beijing, China). All studies were carried out according to an approved protocol of the Institutional Animals Care and Use Committee of the Institute of Zoology, Chinese Academy of Sciences. Adult CD1 virgin females were mated with male mice of the same strain. The morning of a vaginal plug presence was designated as D1. Mice were sacrificed on D1–4 and pregnancy was confirmed by recovering embryos from oviducts or uteri on D1–3 or D4, respectively. The implantation sites on D5–8 were identified by intravenous injection of 1% Chicago blue dye (Sigma–Aldrich, St. Louis, MO) in saline (0.1 ml/mouse) and mice were sacrificed 5 min later. The uteri were cut into 5–8 mm pieces, rapidly frozen in liquid nitrogen and stored at −80°C.

ALKALINE PHOSPHATASE STAINING

The alkaline phosphatase staining process was performed as previously described [Kurihara et al., 2007], with modifications. Briefly, The induced deciduoma and contralateral non-infused horn were fixed in 2% PFA at 4°C, cryoprotected by a sucrose gradient (10%, 20%, 30%, respectively), and embedded in OCT compound (Sakura Finetek, Torrance, CA) for sectioning. Frozen sections (10 μm) were stained with 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and Nitro blue tetrazolium chloride (NBT) solution (Promega, Madison, WI). The development of a purple color was indicative of alkaline phosphatase activity.

IN SITU HYBRIDIZATION

Total RNA of the mouse fibroblast cell line NIH3T3 was extracted and reverse transcribed into cDNA using SuperScript II (Invitrogen, Carlsbad, CA). Smad3 and Smad2 cDNA fragments corresponding to their 3′ untranslated regions were amplified using the following primer pairs: Smad3, forward 5′-GACCTTGGAAGAGAACTCCATC-3′ and reverse 5′-CCAAAGGTAAACTCCATACTCC-3′; Smad2, forward 5′-CAGCACTTGAGGTCTCATCAG-3′ and reverse 5′-CTTGCCTAGACCAAGAAGCAG-3′. The DNA sequences were cloned into pGEM-T vector (Promega) and the authenticity of the DNA were confirmed by sequencing. By using appropriate restriction endonuclease, the plasmids were linearized to serve as the templates. The digoxigenin (DIG)-labeled antisense or sense probes were prepared using DIG RNA labeling kit (SP6/T7; Roche, Mannheim, Germany) according to the manufacturer's protocol.

Frozen sections (10 μm) were mounted onto slides which were coated by 3-aminopropyltriethoxy-silane (Sigma–Aldrich) and fixed in cold 4% paraformaldehyde solution in PBS for 1 h at 4°C. The hybridization process was performed as previously described [Wang et al., 2001], with modifications. Briefly, following pre-hybridization, the sections were hybridized with 1 μg/ml DIG-labeled antisense or sense cRNA probes at 45°C for 16 h. All sections were counterstained with 1% methyl green. Sections hybridized with sense probes, which served as negative controls did not exhibit detectable signals. Three independent mice at each period of pregnancy (D1–8) were examined by in situ hybridization and all experiments were repeated in triplicates.

INDUCED DECIDUALIZATION

Adult WT or Smad3 null mice were ovariectomized and treated with P4 and E2 (Sigma–Aldrich), which mimicked early pregnancy and sensitized the uteri for optimal decidualization [Wordinger et al., 1986]. The ovariectomized animals were arrested for 2 weeks to clear endogenous ovarian hormones and then were treated with a well-established steroid hormone regimen. From days 1 to 3, the mice were daily subcutaneously injected with 100 ng of E2 in 100 μl sesame oil. On days 4 and 5, no hormones were administered. On days 6 and 7, the mice were daily injected with 1 mg P4 and 10 ng E2 in 100 μl sesame oil. From days 8 to 11, the mice were daily injected with 1 mg P4 in 100 μl sesame oil. On day 8, mice were lightly anesthetized and 50 μl sesame oil was delivered intraluminally into one uterine horn, and the noninfused contralateral horn served as a control. On days 12, mice were killed and the control and oil-infused uteri from WT and Smad3 null mice were weighed to assess the extent of decidualization. Localization of Smad2 and Smad3 mRNA was examined in oil-infused and non-infused horns from WT (n = 3) and Smad3 KO mice (n = 3) by using in situ hybridization.

WESTERN BLOTTING

The induced deciduoma and contralateral noninfused horn were quickly collected, frozen in liquid nitrogen, and granulated into fine powder. The tissue powder was homogenized in whole cell lysis buffer (4 mM EGTA, 3 mM EDTA, pH 8.0, 125 mM NaF, 0.5 mM Na_3VO_4, 2.5 μg/ml aprotinin, 25 μg/ml Trypsin inhibitor, 25 μM PMSF, 0.5% NP40, 12.5 mM HEPES, 1 mM DTT). The tissue lysate was centrifuged, and the supernatant was transferred into a new tube. Protein concentrations were determined using a standard Bradford assay, and 50 μg of total protein was separated on a 10% acrylamide gel, and then transferred electrophoretically onto nitrocellulose membranes (Pall Corporation, Pensacola, FL). Membranes were incubated overnight at 4°C with specific primary antibodies against Smad3, Smad2, phospho-Smad2 (Cell Signaling Technology, Beverly, MA) and β-actin (Santa Cruz, Santa Cruz, CA), followed by incubation with appropriate secondary antibody. The blot was developed using the Enhanced Chemiluminescence System (Pierce, Rockford, IL). Samples from WT (n = 6) and Smad3 KO mice (n = 6) were subjected to Western blotting analysis, which was repeated in triplicates. Bands intensity was quantified by densitometry using Quantity One software (Bio-Rad, Munich, Germany).

PRIMARY CULTURE OF UTERINE STROMAL CELLS

The mouse uterine stromal cells were isolated following the procedure previously described [Kimura et al., 2001; Tan et al., 2004] with some modifications. Briefly, the wide-type or Smad3 null mice were ovariectomized and arrested for 2 weeks to clear endogenous ovarian hormones and then were treated as follows: E2 (100 ng) for 3 days (days 1–3), no treatment on days 4 and 5, P4 (1 mg) + E2 (10 ng) on days 6 and 7. On day 8, the uterine horns were collected and dissected longitudinally to expose the uterine lumen and cut into pieces. The uterine pieces were washed twice with Hank's balanced salt solution (HBSS) containing 100 U/ml penicillin and 100 μg/ml streptomycin (Gibco-BRL, Gaithersburg, MD) before being incubated for 1 h at 4°C in 2 ml HBSS containing antibiotics, 0.25% pancreatin (Sigma–Aldrich) and 6 mg/ml dispase (Gibco-BRL), and followed by 1 h at room temperature, and 10 min at 37°C. The digestion steps were immediately terminated by adding 2 ml HBSS containing 10% charcoal-stripped fetal bovine serum (FBS), and the epithelial cells were eliminated by pipetting up and down. The remaining tissues were washed twice with HBSS containing antibiotics and incubated in 2 ml HBSS containing 0.5 mg/ml collagenase Type II (Invitrogen) for 30 min at 37°C, and then the tissues were diluted in HBSS containing 10% charcoal-stripped FBS and pipetted up and down thoroughly until the supernatant became turbid. The supernatants were transferred into a new tube and centrifuged at 1,000 rpm for 10 min. The supernatants were discarded and the precipitates were resuspended in 5 ml HBSS. The supernatants mainly containing stromal cells were passed through a 70 μm nylon filter and then centrifuged at 1,000 rpm for 10 min. The pellet was washed twice with Dulbecco's modified Eagle's Medium-F12 medium (DMEM/Ham's F-12, 1:1) before the initiation of primary culture. 4×10^5 cells were seeded in a 6-well plate, containing phenol red-free culture medium (DMEM/Ham's F-12, 1:1) with 10% charcoal-stripped FBS and antibiotics. The unattached cells were removed by washing with DMEM/Ham's F-12 medium after incubation for 2 h, and fresh medium (DMEM/Ham's F-12, 1:1) containing 1% chacoal-stripped FBS, antibiotics, 10 nM E2, and 1 μM P4 was added to the attached cells.

IMMUNOCYTOCHEMISTRY

The cells were fixed in ice-cold methanol for 10 min and permeabilized for 7 min with 0.01% Triton X-100 in PBS. After incubation with blocking buffer (3% bovine serum albumin), cells were incubated with primary antibodies against vimentin (ab20346, Abcam, Cambridge, MA) and cytokeratin (ab6401, Abcam). Cells were washed with PBS and incubated with appropriate fluorescein isothiocyanate (FITC)-conjugated secondary antibodies (Zhongshan Corp, Beijing, China). Nuclei were counterstained with propidium iodide (PI). Stained cells were analyzed by a fluorescence microscope.

RNA PREPARATION AND QUANTITATIVE REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (qRT-PCR)

Total RNA was extracted using Trizol reagent (Invitrogen) according to the manufacturer's protocol. Two micrograms of total RNA were reverse transcribed into single-stranded cDNA by using Superscript II reverse transcriptase (Invitrogen). cDNA was amplified using SYBR® Premix Ex Taq™ (Perfect Real Time) reagent (Takara, Dalian, China) according to the manufacturer's instructions. Quantitative PCR was performed with ABI PRISM 7500 Sequence Detection System (Applied Biosystems, Foster City, CA). The results were analyzed using ΔCt method by using ABI Prism 7500 SDS Software. The data were normalized to GAPDH expression. Primers used for quantitative PCR were as follows: Smad2, forward 5′-GCAAATACGGTAGATCAGTGGG-3′ and reverse 5′-CAGTTTTC-GATTGCCTTGAGC-3′; Smad3, forward 5′-CCCCCACTGGAT-GACTACAG-3′ and reverse 5′-TCCATCTTCACTCAGGTAGCC-3′; dPRP, forward 5′-TTATGGGTGCATGGATCACTCC-3′ and reverse 5′-CCCACGTAAGGTCATCATGGAT-3′; cyclin D3, forward 5′-GCTCCAACCTTCTCAGTTGC-3′ and reverse 5′-AGCTAAGCAG-

CAAGCAAAGC-3′; GAPDH, forward 5′-GGTGAAGGTCGGTGT-GAACG-3′ and reverse 5′-CTCGCTCCTGGAAGATGGTG-3′.

RNA INTERFERENCE

Small interfering RNAs (siRNAs) were designed by Genepharma (Shanghai, China). The sequence of siRNAs corresponding to mouse Smad2 is located within exon 5: 5′-GAGCCACAGAGUAAUUA-CATT-3′. A universal control siRNA was used as a nonspecific control. siRNA duplex was transfected into primary stromal cells with Lipofectamine® 2000 (Invitrogen) as recommended by the manufacturer.

STATISTICAL ANALYSIS

Values were illustrated as means ± SEM of at least three independent experiments. The significance of difference was assessed by unpaired *t*-test. $P < 0.05$ was considered statistically significant ($^{*}P < 0.05$, $^{**}P < 0.01$). Statistical analysis was conducted with SPSS 17.0 software (SPSS Software, Chicago, IL).

RESULTS

Smad3 IS EXPRESSED IN A SPATIOTEMPORAL MANNER DURING MOUSE PERI-IMPLANTATION PERIOD

To evaluate the physiological relevance of Smad3 in early pregnancy events, we first examined the expression of Smad3 in the endometrium during early pregnancy in mice. Smad3 mRNA expression was extremely low in the endometrium from D1 to 4, excepting a low expression in the luminal epithelium and glandular epithelium on D3 (Fig. 1A–D), but was significantly up-regulated with the onset of implantation on D5. Smad3 was also highly expressed in the stromal cells surrounding the blastocyst on D5 (Fig. 1E). As shown in Figure 1E–H, the expression of Smad3 was highly coincident with the decidual transformation. On D6, Smad3 was highly expressed in the continually proliferating SDZ, but it was not expressed in the PDZ (Fig. 1F). On D7 and 8, Smad3 remained an abundant expression in the decidual zone (Fig. 1G,H). From the longitudinal sections, we found that Smad3 was specifically expressed in the stromal cells surrounding the blastocyst and the decidual zone (Fig. 1I–K). Uterine cross sections hybridized with the sense probe did not show any specific positive signals (Fig. 1L).

Smad3 NULL MICE SHOW COMPROMISED DECIDUALIZATION

To address whether Smad3 is involved in the process of decidualization, we next examined the decidualization in Smad3 deficient mice. As shown in Figure 2A,D, decidualization could be induced in both WT ($Smad3^{+/+}$) and Smad3 deficient ($Smad3^{-/-}$) mice. Alkaline phosphatase, which has strong activities in the antimesometrial decidual cells and weak staining in the mesometrial region [Smith, 1973; Lee et al., 2006], could be detected in artificially induced deciduoma of both $Smad3^{+/+}$ and $Smad3^{-/-}$ mice (Fig. 2B,E), indicating that the decidual reaction could be

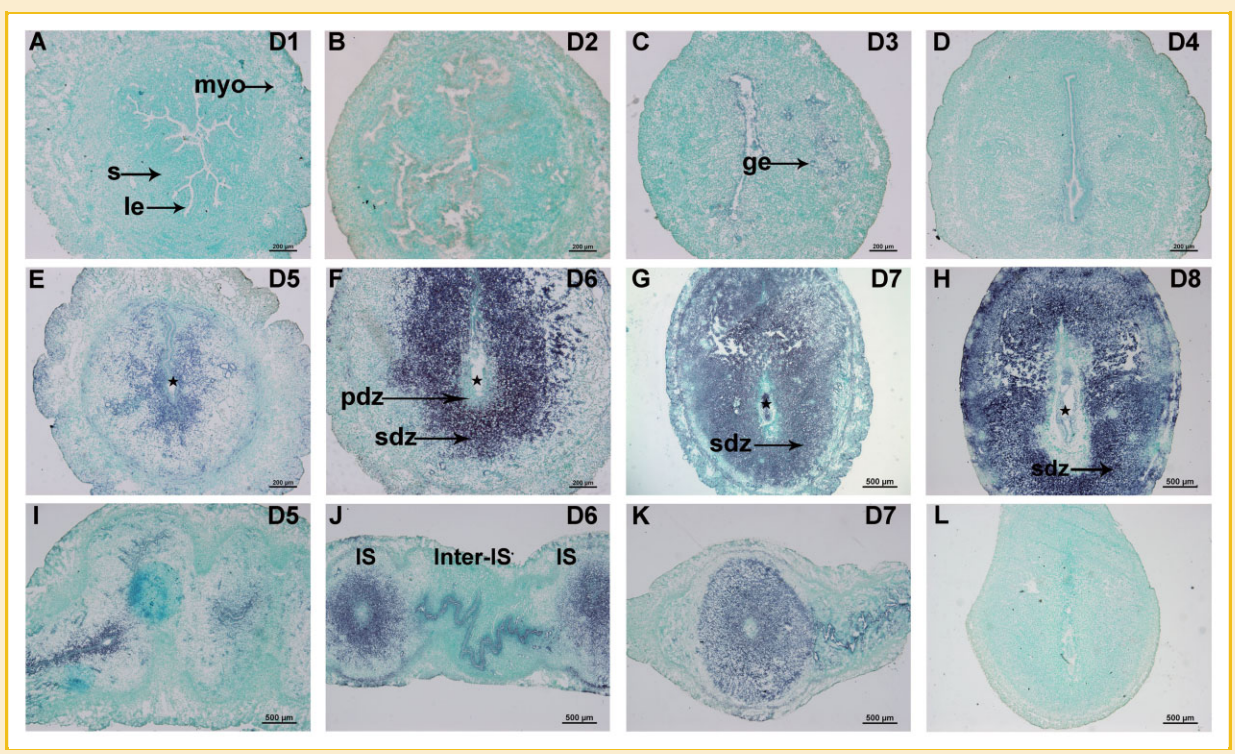

Fig. 1. Smad3 mRNA was expressed in a spatiotemporal manner during the peri-implantation period in mouse endometrium. A–H: In situ hybridization of Smad3 mRNA in the cross sections of uteri during peri-implantation period. I–K: In situ hybridization of uterine Smad3 in the longitudinal sections from D5 to 7. L: Hybridization with Smad3 sense probe. Nuclei were lightly counterstained with methyl green. ★ indicates the location of embryo. le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium; pdz, primary decidual zone; sdz, secondary decidual zone; D, day of pregnancy. IS, implantation site; Inter-IS, interimplantation site.

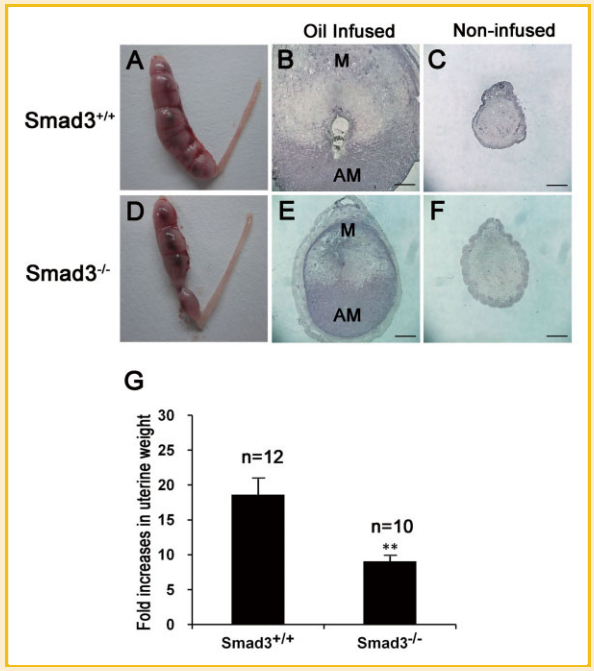

Fig. 2. Smad3 null mice showed compromised decidualization. A,D: Representative uterine horns with artificially induced decidualization in WT (Smad3$^{+/+}$) and Smad3 null (Smad3$^{-/-}$) mice. Alkaline phosphatase activity was detected in the oil-induced deciduoma of Smad3$^{+/+}$ (B) and Smad3$^{-/-}$ (E) mice and the contralateral noninfused horn (C,F) 4 days after the decidual stimulus. AM, antimesometrial pole; M, mesometrial pole. Bars = 500 μm. G: The extent of decidualization was determined by the fold increases in wet weight between the oil infused uterine horn and the contralateral noninfused horn. Bar graph represents mean ± SEM. $^{**}P < 0.01$, student's *t*-test. Numbers above the bar indicate the number of animals used.

induced. In the stromal cells of contralateral non-infused horns, activities of alkaline phosphatase were absent (Fig. 2C,F). We evaluated the extent of decidualization by comparing fold increase in uterine wet weights of the infused horn compared with noninfused horn. The extent of decidualization in Smad3$^{-/-}$ mice was significantly compromised, relative to Smad3$^{+/+}$ mice. Smad3$^{+/+}$ mice showed an average of 18-fold increase in uterine weight in the oil-infused horn compared with noninfused horns, whereas the Smad3$^{-/-}$ mice showed an average of nine fold increase in uterine weight ($P < 0.01$, Fig. 2G).

Smad2 AND Smad3 ARE CO-LOCALIZED IN THE DECIDUAL ZONE DURING MOUSE PERI-IMPLANTATION

As either Smad2 or Smad3 serves as intracellular R-Smad of TGF-β/activin signals, we speculate that Smad2 may compensate for the loss of Smad3 during the decidualization of Smad3 deficient mice. Therefore, we first detected the in situ localization of Smad2 mRNA during the peri-implantation period. As shown in Figure 3A–D, we could detect the expression of Smad2 in the glandular epithelium on D1–4. On D5, Smad2 was expressed in stromal cells around the blastocyst (Fig. 3E). From D6 to 8, Smad2 was highly expressed in the SDZ (Fig. 3F–H). These data confirm that Smad2 and Smad3 are co-localized in the decidual zone.

Smad3 AND Smad2 ARE BOTH HIGHLY UP-REGULATED IN ARTIFICIALLY INDUCED DECIDUOMA AND THE EXPRESSION OF PHOSPHORYLATED Smad2 IS HIGHER IN THE DECIDUOMA OF Smad3 NULL MICE THAN THAT IN WT MICE

We further utilized in situ hybridization to detect the expression of Smad3 and Smad2 mRNA in artificially induced deciduoma, and found that both Smad3 and Smad2 were abundantly expressed in the deciduoma of WT mice (Fig. 4A,D), and that Smad2 was also highly induced in the deciduoma of Smad3$^{-/-}$ mice (Fig. 4G). In the contralateral non-infused horn of WT mice, Smad3 mRNA was expressed in the endometrial epithelium and stromal cells next to the epithelium (Fig. 4B), and a similar localization pattern was observed for Smad2 mRNA in the contralateral non-infused horn of both WT and Smad3$^{-/-}$ mice (Fig. 4E,H).

We further employed Western blotting to detect the expression of Smad3, Smad2 and phosphorylated Smad2 in the induced deciduoma and contralateral noninfused horn. As shown in Figure 4J–L, both total Smad2 and phosphorylated Smad2 were significantly increased in the deciduoma compared with the contralateral noninfused horn in WT and Smad3 null mice. Expression of total Smad2 did not show any statistically significant difference between the WT and Smad3 null mice (Fig. 4J,L), both in infused deciduoma and non-infused horns, but the phosphorylated Smad2 was much more abundantly increased in the deciduoma of Smad3 null mice (Fig. 4J,K). We also found that Smad3 was up-regulated in the WT deciduoma as compared with the contralateral noninfused horn (Fig. 4J,M).

Smad2 AND Smad3 MAY HAVE REDUNDANT FUNCTION DURING THE DECIDUALIZATION OF PRIMARY CULTURED STROMAL CELLS

In order to investigate the mutually compensatory roles of Smad2 and Smad3 during the process of decidualization, we employed an in vitro uterine stromal cell decidualization model. The purity of isolated uterine stromal cells was determined by immunofluorescence staining of vimentin and cytokeratin. Over 95% of the isolated cells were identified as vimentin-positive and cytokeratin-negative stromal cells, as shown in Figure 5A,B. We also validated the in vitro decidualization by detecting the expression of dPRP, a well-known differentiation marker of uterine stromal cells [Rasmussen et al., 1997]. As shown in Figure 5C, the expression of dPRP was intensely up-regulated from 0 to 72 h of culture.

We detected the expression of Smad3 and Smad2 in WT stromal cells using the method of qRT-PCR, and found that both Smad3 and Smad2 were up-regulated along with the culture time from 0 to 72 h (Fig. 5D,E), which was consistent with the in vivo expression pattern of Smad3 and Smad2 as previously shown in Figures 1 and 3. We further applied siRNA to knockdown the expression of Smad2 in WT and Smad3 null stromal cells. As shown in Figure 5F, the knockdown efficiency of Smad2 mRNA was confirmed in decidual cells. We further examined the expression of decidualization marker gene, dPRP and decidualization related gene, cyclin D3. After knocking down the expression of Smad2 in WT decidual cells, the expression of dPRP remained statistically unchanged (Fig. 5G). However, the relative expression of dPRP was significantly down-regulated in the Smad3 null decidual cells than for WT decidual cells (Fig. 5H, $P < 0.05$). Notably, when Smad2 was simultaneously

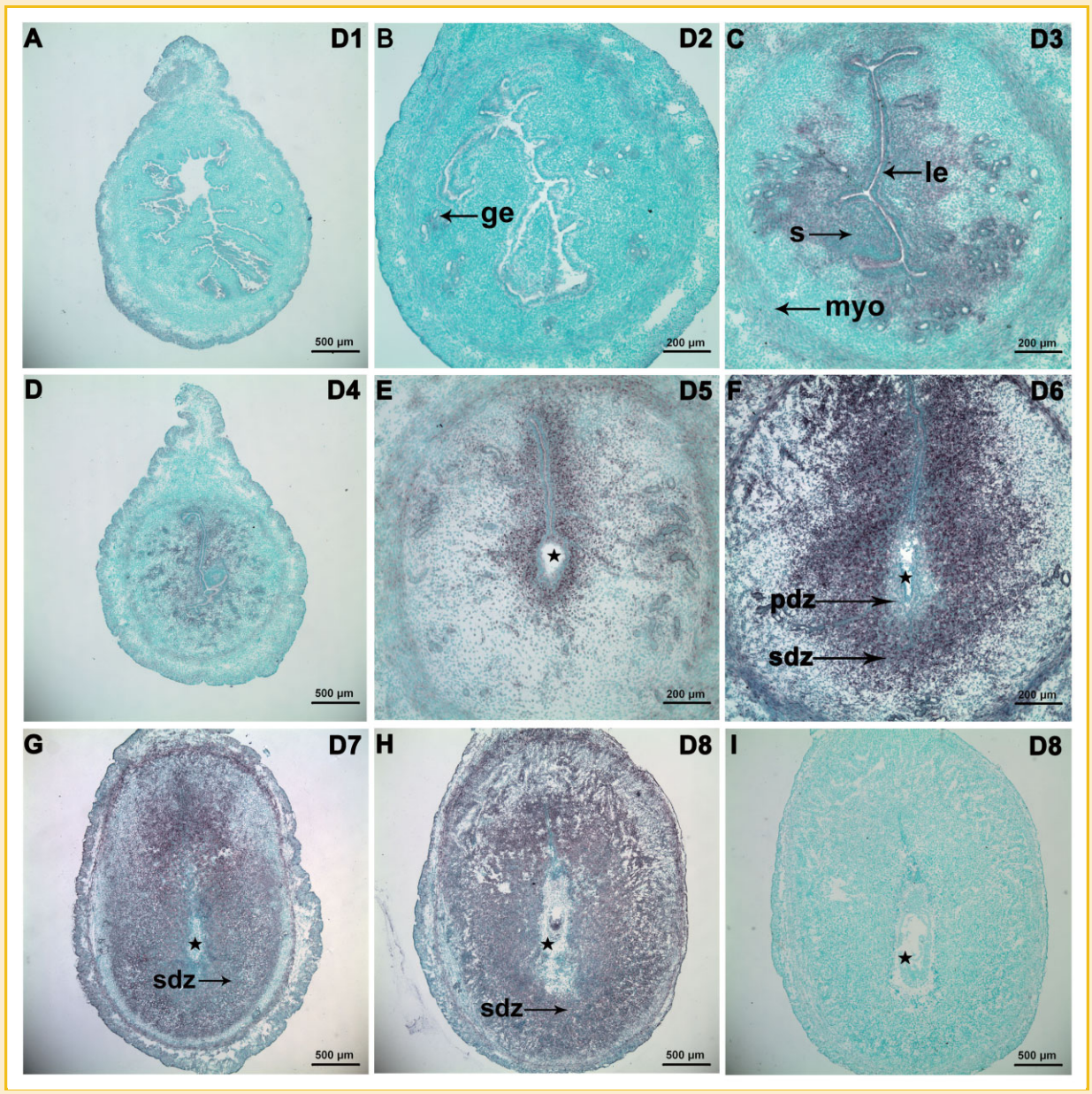

Fig. 3. Smad2 mRNA was expressed in the decidual zone and luminal epithelium in the peri-implantation mouse uterus. A–H: In situ hybridization of Smad2 mRNA in the cross-sections of mouse uteri at indicated stages of pregnancy. I: Hybridization with Smad2 sense probe. The sections were lightly counterstained with methyl green to show the nuclei. ★ indicates the location of embryo. le, luminal epithelium; ge, glandular epithelium; s, stroma; myo, myometrium; pdz, primary decidual zone; sdz, secondary decidual zone; D, day of pregnancy.

silenced in Smad3 null decidual cells, dPRP was much more significantly reduced (Fig. 5I, $P < 0.05$). The expression of cyclin D3 showed similar pattern as that of dPRP (Fig. 5J–L). We also detected other decidualization-related genes including CDK1, CDK2, CDK4, CDK6, and p21, and found no significant changes (data not shown).

DISCUSSION

During mouse embryo implantation, after the apposition process, the blastocyst attaches to the uterine luminal epithelium, and then stromal cells surrounding the blastocyst proliferate and differentiate, forming decidua [Abrahamsohn and Zorn, 1993]. The decidualization of uterine stromal cells protects the embryo from maternal immunocompetent cells [Wang et al., 2004], and regulates the invasion of trophoblast cells [Tranguch et al., 2005]. Accordingly, successful decidualization is a crucial process in embryo implantation. The decidualization is a dynamic and complex process during which cellular proliferation, differentiation and apoptosis occur in a spatiotemporal manner. In our study, we observed that both Smad3 and Smad2 were highly expressed in the mouse decidual zone during the peri-implantation period and

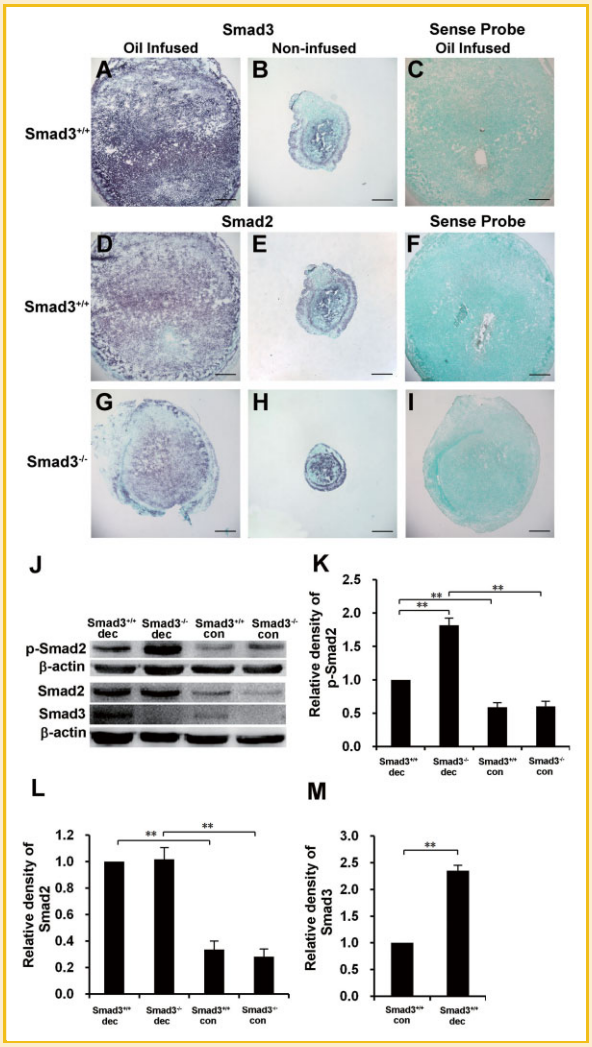

Fig. 4. Smad3 and Smad2 were abundantly expressed in the artificially induced deciduoma and phosphorylated Smad2 was significantly increased in Smad3 null mice deciduoma. A–I: In situ hybridization of Smad3 mRNA in the cross section of $Smad3^{+/+}$ deciduoma (A) and Smad2 mRNA in $Smad3^{+/+}$ (D) and $Smad3^{-/-}$ (G) deciduoma and the contralateral non-infused horns (B,E,H). C,F,I: Hybridization with Smad3 or Smad2 sense probes. Bars = 500 μm. J: Western blotting analysis of phosphorylated Smad2, total Smad2 and Smad3 in the artificially induced deciduoma and the contralateral noninfused horns of WT and Smad3 null mice. K–M: The protein levels of phosphorylated Smad2, total Smad2, and Smad3 were quantitated by analyzing densitometric readings of each band and normalized with beta-actin protein level. The differences were determined by student's *t*-test ($^{**}P < 0.01$). $Smad3^{+/+}$ dec, experimental induced deciduoma from WT mice. $Smad3^{-/-}$ dec, experimental induced deciduoma from Smad3 null mice. $Smad3^{+/+}$ con, noninfused contralateral horn from WT mice. $Smad3^{-/-}$ dec, noninfused contralateral horn from Smad3 null mice. β-actin was included as a loading control.

Smad3 deficient mice showed compromised decidualization. In vitro studies suggested that Smad3 and Smad2 might have redundant roles during the process of decidualization.

Previous studies have shown that many of the TGF-β superfamily members are expressed in the uterus. In the mouse, TGF-β1 is expressed in the secondary decidual zone and TGF-β2 is detected in the decidual cells from D5 to 8 [Das et al., 1992; Manova et al., 1992; Cheng et al., 1993]. On the other hand, activin βA and βB subunits are detected from D5 to 8 in the decidual cells [Manova et al., 1992]. Both TGF-β and activin are transduced through intracellular Smad3 and Smad2, and our results showed a consistent spatio-temporal expression of Smad3 and Smad2 with that of TGF-β and activin in decidual cells. We found that Smad3 and Smad2 were highly expressed in the stromal cells that formed the PDZ on D5, and from D6 to 8, while Smad3 and Smad2 were highly expressed in the SDZ instead of the PDZ. The expression patterns of Smad3 and Smad2 indicate that Smad3 and Smad2 may mediate the signals from TGF-βs and activins during mouse decidualization. TGF-β1 and TGF-β2 are expressed in the luminal and glandular epithelia on D1–4 [Tamada et al., 1990; Das et al., 1992]. However, in the current study, Smad3 expression was very low in the mouse uterus on D1–4, with a slight expression on D3, but we observed Smad2 expression in endometrial epithelium from D1 to 4. This suggests that TGF-βs may function through Smad2 instead of Smad3 during the pre-implantation phases. The spatiotemporal expression manners of Smad3 and Smad2 indicate that maybe they played different or redundant roles at specific stages of early pregnancy and they mediate signals from distinct ligands of TGF-β subfamily members in specific cell types and stages in the mouse uterus. This is supported by the expression patterns of Smad2 and Smad3 in the rat testis, where Smad2 and Smad3 are often but not always expressed in the same cell type, indicating developmental and stage specific responses of either Smad to activin and TGF-β [Xu et al., 2003].

There is no direct genetic evidence indicating that the TGF-β signaling pathway affects decidualization in mice, in part because TGF-β1, TGF-β2, TGF-β3, Nodal and activin null mice are either perinatal lethal or viable and fertile [Chang et al., 2002]. However, despite the finding that TGF-β and activin play essential roles in human endometrium decidualization in vitro [Jones et al., 2002; Kim et al., 2005; Chang et al., 2008], the mechanism underlying how these pivotal growth factors are involved in decidualization remains poorly understood. The specific and abundant expression of Smad3 and the co-localization with TGF-β and activin in the mouse decidual zone led us to hypothesize that Smad3 may play an important role during the decidualization process. To address this hypothesis, we employed the Smad3 knockout mice to elucidate the function of Smad3 in the decidualization.

Smad3 null mice are smaller prior to weaning and many survive until 8 months old, but are infertile [Yang et al., 1999]. This is attributed to impaired folliculogenesis and defects in ovulation [Tomic et al., 2002]. We therefore employed an artificial decidualization animal model, which has been widely used to study the mechanisms of decidualization [Lim et al., 1997; Das et al., 2009]. In pregnant mice, the stimulus for decidualization is the implanting blastocyst. By intraluminal oil infusion, the decidualization process can be artificially induced in pseudopregnant or steroid hormonal prepared mice [Finn and Martin, 1972; Kennedy, 1983]. In the mouse, P4, which is secreted from the newly formed corpora lutea, plays an important role in decidualization [Lydon et al., 1995]. In the Smad3 null mice, P4 is insufficient due to little corpora lutea. To circumvent the steroid deficiency, we examined the decidualiza-

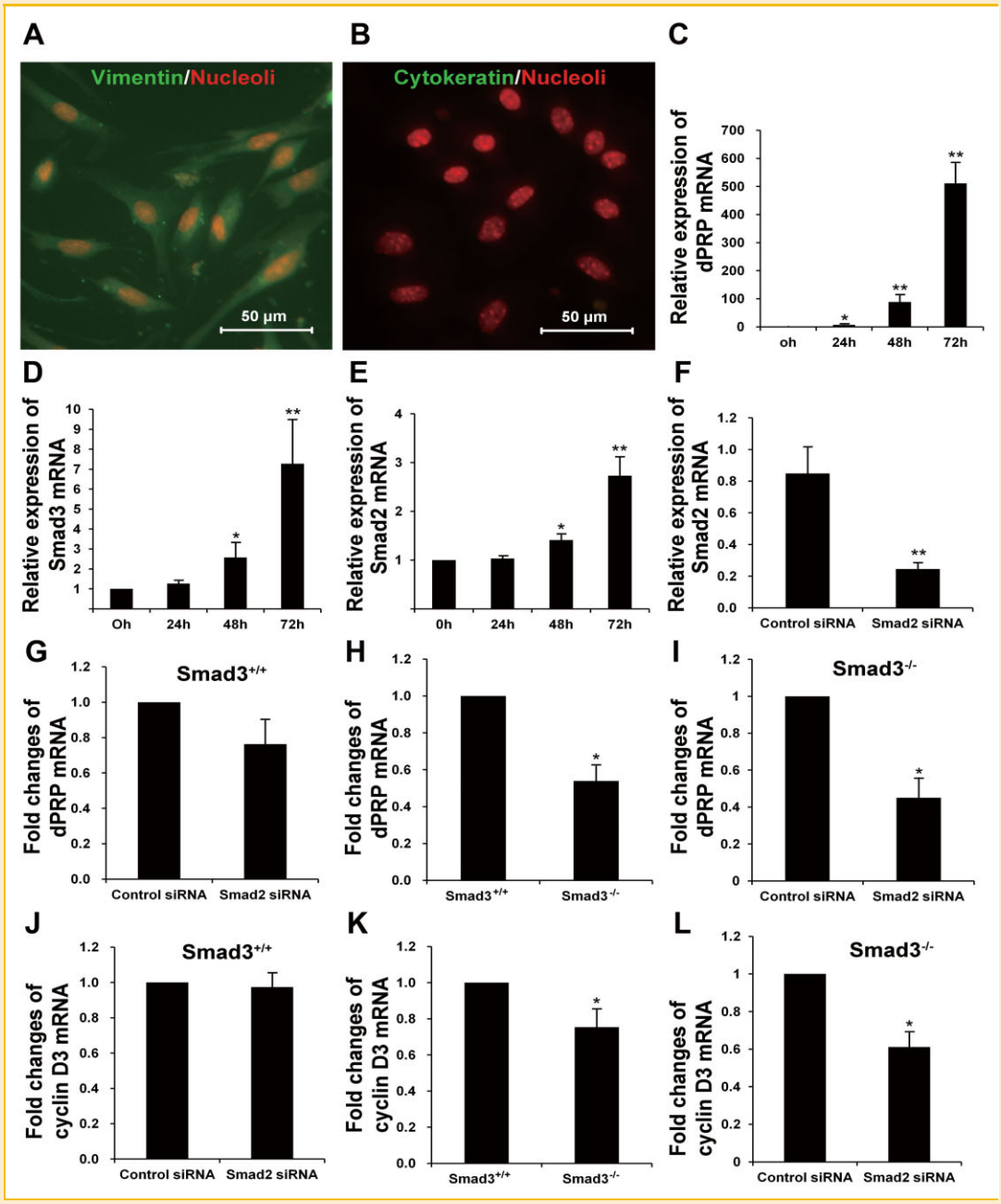

Fig. 5. Smad2 and Smad3 might function redundantly during in vitro decidualization. Isolated mouse uterine stromal cells were cultured in the presence of E2 and P4 and underwent in vitro decidualization. A,B: The primary cultured stromal cells were identified by immunofluorescence staining of vimentin (A) but not cytokeratin (B). The ratio of vimentin-positive cells to total cells was more than 95%. C: The expression of dPRP, a sensitive marker of stromal cell decidualization, was determined by qRT-PCR at different time point of in vitro decidualization. GAPDH was used as an internal reference gene for normalization. The graph showed the relative expression level of dPRP compared with that at 0 h. D,E: The fold changes of Smad3 and Smad2 mRNAs compared with those at 0 h were shown. Smad2 and Smad3 were up-regulated during the process of in vitro decidualization as determined by qRT-PCR. F: The efficiency of siRNA-mediated knockdown of Smad2 determined by qRT-PCR. mRNA expression of dPRP (G–I) and cyclin D3 (J–L) in Smad3 null decidual cells (H,K) and when knocking down Smad2 in WT (G,J) and Smad3 null decidual cells (I,L) was determined by qRT-PCR (student's *t*-test; $^{*}P < 0.05$, $^{**}P < 0.01$).

tion process in Smad3 null mice by employing a steroid hormonal prepared model, as previously described [Lim et al., 1997; Kurihara et al., 2007]. We observed that in the absence of Smad3 the decidualization was compromised, indicating that Smad3 was essential for mouse uterine decidualization.

Although the decidualization was compromised in the Smad3 null mice, the decidualization could still occur. We speculated that Smad2, which was co-expressed with Smad3 in the mouse decidual zone (Fig. 3E–H), could partially compensate for the loss of Smad3 during the process of decidualization. Smad2 and Smad3 share 92%

identity in amino acid sequences [Dennler et al., 1999], and they have redundant function in various physiological process including mouse ovarian cumulus cell expansion [Li et al., 2008], IL-17-producing (Th17) cells development in vitro [Lu et al., 2010] and long bone development [Alvarez and Serra, 2004]. To investigate the possible redundant function of Smad2 and Smad3 during decidualization, we utilized in vivo artificially oil-induced decidualization model to detect the expression pattern of Smad2 and Smad3 in deciduoma from WT and Smad3 null mice.

The nonspecific stimulus, intraluminal infusion of oil, could evoke certain aspects of decidual cell reaction in steroid hormonally prepared uteri [Dey et al., 2004]. By using in situ hybridization, we observed that Smad2 mRNA was co-localized with Smad3 in the decidual zone of artificially induced deciduoma, resembling the localization of physiological decidualization, suggesting that they might function similarly during normal physiological decidualization and oil-induced decidualization. Consistent with the in situ hybridization results, Western blotting analysis also illustrated the up-regulation of total Smad2 and Smad3 in WT deciduoma, and total Smad2 in Smad3 null deciduoma, compared with the corresponding contralateral non-infused horn. Remarkably, phosphorylated Smad2 was also highly induced in both WT and Smad3 null deciduoma whereas it was much more significantly increased in the deciduoma of Smad3 null mice than for wild-type (WT) mice. The even more abundant up-regulation of phosphorylated Smad2 in the deciduoma of Smad3 null mice indicate that Smad2 might function redundantly during decidualization and when Smad3 is ablated Smad2 could be overtly activated to compensate for the function of Smad3.

We further utilized in vitro uterine stromal cell decidualization model which has been widely used to investigate the mechanism of decidualization [Kimura et al., 2001; Chen et al., 2009] to investigate the possible redundancy of Smad3 and Smad2. Both Smad3 and Smad2 were up-regulated during the in vitro decidualization process, a pattern similar to that during physiological and artificially induced decidualization. The expression of decidualization markers dPRP and cyclin D3 [Rasmussen et al., 1997; Das et al., 1999] in WT decidual cells were not significantly influenced by knocking down Smad2, but were significantly down-regulated in the Smad3 null decidual cells than for WT decidual cells. Interestingly, when Smad2 was simultaneously silenced in Smad3 null decidual cells, the two markers were much more significantly reduced, providing additional in vitro evidence that Smad2 and Smad3 might function redundantly.

In summary, the present study has demonstrated that Smad3 was highly expressed in the mouse decidual zone during the peri-implantation period and Smad3 null mice showed compromised decidualization. Smad2 and Smad3 were co-localized in the mouse decidual zone and they might function redundantly during the mouse decidualization process. Our study provides the first genetic evidence for the essential function of Smad3 during mouse decidualization.

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