## DISCUSSION

In this study, we disrupted the gene for mROCK-II, one of the two isoforms of ROCK, and found that most ROCK-II-deficient homozygous mice died in utero. A small number of ROCK-II$^{-/-}$ mice that survived were born runts. Although two ROCK-II$^{-/-}$ mice that displayed severe growth retardation died within 4 weeks, most subsequently developed apparently normally. These results indicated that the embryonic lethality found in these mice was caused by a defect(s) not in the embryo per se but in the embryo-placenta interaction. We could place this defect in the labyrinth layer of the placenta. First, using the expression of the mROCK-II-*lacZ* reporter gene, we found that trophoblasts in this layer highly expressed ROCK-II. We further found that marked thrombosis and the progressive loss of trophoblasts occurred in the labyrinth layer of ROCK-II$^{-/-}$ mice but not in that of wild-type or ROCK-II$^{+/-}$ heterozygous mice.

The question then is how the loss of ROCK-II induces this pathology of the labyrinth layer. Defects in the development of the labyrinth layer and resultant embryonic death have been found in a number of strains of genetically engineered mice (10). However, no histological abnormality was observed in ROCK-II$^{-/-}$ placentas before 12.5 dpc. The phenotype of ROCK-II$^{-/-}$ mice is therefore different from the labyrinth dysplasia found in strains with mutations in the genes encoding fibroblast growth factor receptor 2 (38), hepatocyte growth factor (33), adapter proteins Grb2 (27) and Gab1 (15), Sos1 Ras exchanger (25), Mek1 (8), p38α (1), and Mekk3 (40) and suggests that ROCK-II is not involved in the initial development of the placenta (35). The placenta proper containing the labyrinth layer is fully formed and functional by 12 dpc, and the volume of the labyrinth layer increases to about half of the total placental volume between 12 and 17 dpc (2). During this gestation period, the perfusion pressure of the maternal blood flowing in the labyrinth layer rises and then remains high. Both the volume expansion and the increase in perfusion pressure are necessary to support the drastic growth of the embryo. Because failure of ROCK-II$^{-/-}$ embryos occurs in this period, it is likely that ROCK-II works at this stage of maturation and adaptation of the labyrinth layer. Several adaptive responses could be required. One may be resistance of the labyrinth structure to the shearing force of the blood flow, and the other may be protection from coagulation and thrombosis. The labyrinth sinusoid where maternal blood flows is lined not by endothelium but by special types of trophoblasts (2). ROCK-II is probably involved in such adaptive changes in the trophoblasts in response to blood flow, because they highly express ROCK-II (Fig. 4B and D).

We examined possible defects in the adaptive responses of trophoblasts in the placentas of ROCK-II$^{-/-}$ mice in two ways. First, we examined the possibility that loss of mROCK-II affected the cytoarchitecture of the labyrinth layer by staining F-actin both in tissues and in cultured trophoblasts. No significant differences in the structure of actin cytoskeleton and the strength of phalloidin staining were found in mutant and wild-type tissues and cells. The actin bundling structure in cultured ROCK-II$^{-/-}$ trophoblasts was also sensitive to Y-27632, suggesting that ROCK-I maintains the actin structure in the labyrinth layer in the absence of ROCK-II. However, this does not necessarily exclude the possibility that both ROCK-I and -II are required for proper function of actin structures (e.g., to maintain tissue integrity or to exert contractility in response to stimuli). This issue remains to be investigated. Second, we performed a microarray analysis and examined possible changes of gene expression in the placentas of ROCK-II$^{-/-}$ mice. By this analysis, we detected higher levels of expression of at least three genes, including the prolactin-like protein G and PAI-1 genes (Table 2). We further confirmed the elevated expression of PAI-1 both by RT-PCR and Northern blotting (Fig. 7). Because PAI-1 inhibits fibrinolytic activity of tissue plasminogen activator (4), elevation of PAI-1 expression could very well lead to an enhanced coagulation tendency (29, 39). Indeed, elevated expression of PAI-1 in the placenta has been reported in patients with gestational trophoblastic disease and is proposed to contribute to the hemostatic problems associated with this disorder (6). In addition, an increase in PAI-1 activity of a few-fold could be responsible for the thrombogenic tendency in experimental animals (30). These results therefore suggest that the thrombus formation in the labyrinth layer of the ROCK-II$^{-/-}$ placenta is caused at least partly by enhanced expression of PAI-1. In addition to PAI-1, increased expression of prolactin-like protein G may also lead to the thrombotic tendency. This protein belongs to the family of prolactin-like proteins and is most homologous to prolactin-like proteins E and F (21). While the activity of prolactin-like protein G remains to be tested, prolactin-like proteins E and F are known to stimulate proliferation and differentiation of megakaryocytes via the specific receptor-gp130 pathway to increase the number of platelets in the circulation system (41). It is therefore likely that elevated expression of these genes enhanced the thrombogenic tendency in the ROCK-II$^{-/-}$ placenta. At present, we do not know whether the expression of these genes is a direct consequence of the loss of ROCK-II or is triggered by unknown primary events directly influenced by the loss of this enzyme. In contrast to the findings of this study, previous experiments using Y-27632 in cultured smooth muscle cells and an experimental animal suggest that ROCK mediates the angiotensin-induced PAI-1 expression (16, 32). Whether the PAI-1 expression is a direct or indirect consequence, elevated expression of this gene probably contributes to the induction of the phenotype of ROCK-II$^{-/-}$ mice, given the potent thrombogenic action of this substance.

Elevated expression of PAI-1 in the placentas of ROCK-II$^{-/-}$ mice may also explain the hemorrhages observed in ROCK-II$^{-/-}$ embryos. These hemorrhages, apparently caused by dilation of peripheral capillaries, were found in the hind limbs and the tip of the tails of the embryos, resolved, and did not recur in adult mice. Interestingly, a similar but more severe phenotype was observed in PAI-1 transgenic mice (5), which showed subcutaneous hemorrhage at the tip of the tail and edema of the hind paw. The researchers attributed these symptoms to downstream venous occlusion. Given the elevated expression of PAI-1 in ROCK-II$^{-/-}$ placenta, we examined PAI-1 expression by RT-PCR in the whole embryo at dpc 13.5. However, we could not detect an amplified product (data not shown), suggesting that PAI-1 is not expressed or expressed at a very low level in organs other than the placenta at this embryo stage. It is therefore plausible that PAI-1 generated in the placenta was transported to the fetal circulation system and

caused occlusion of particular veins in ROCK-II$^{-/-}$ mice. Such PAI-1 action, if present, may explain the transient nature of the hemorrhage.

Rho and ROCK work downstream of extracellular signaling, and a number of molecules and conditions induce activation of Rho. The question then is what kinds of molecules and conditions induce Rho activation in the labyrinth layer. One of the signaling pathways activating Rho and ROCK is the Wnt-Frizzled pathway (37). It is interesting that mice deficient in Wnt2 showed a placental dysfunction (20) similar to those we found in ROCK-II$^{-/-}$ mice. Wnt2$^{-/-}$ mice were born runts and exhibited an embryonic lethality of 50%. Histological examination showed that about 85% of Wnt2$^{-/-}$ placentas showed defects in the labyrinth layer, including accumulation of abnormally large hematomas and fibrinoid materials. These findings suggest a possibility that ROCK-II works in the Wnt2 pathway in maturation and adaptation of the labyrinth layer in the late stage of gestation.

We thus identified malfunction of the labyrinth layer as the cause of embryonic lethality and growth retardation in ROCK-II$^{-/-}$ mice. While the labyrinth layer malfunction is a major phenotype of ROCK-II deficiency, we do not know whether this phenotype is induced by suppression of actions specific for ROCK-II or suppression to some extent of the combined actions of ROCK-I and -II. Besides this intrauterine phenotype, most ROCK-II$^{-/-}$ mice born alive developed and grew without obvious anatomical and functional abnormalities. This is rather surprising given the critical functions ROCK exerts in organization of the actin cytoskeleton in various types of cells and the different, albeit partially overlapping, patterns of tissue expression of the two isoforms. This is more surprising in the light of the finding that ROCK-I did not increase to compensate for the loss of ROCK-II at least in the embryo, placenta, and brain of adult mice (Fig. 2 and Table 2). It is of course possible that there are abnormalities in ROCK-II-deficient mice, which can be revealed only by detailed functional and histological analyses. This issue will be examined by future studies. The ROCK-II$^{-/-}$ mouse phenotype, including enhanced PAI-1 expression, is strongly reminiscent of human IUGR, for which thrombosis in the placenta is proposed as a major cause (9, 23). IUGR comprises a significant fraction of prenatal morbidity and low-birth-weight babies (29). Understanding molecular mechanisms of the failure of ROCK-II$^{-/-}$ placenta in more detail may provide insight into the physiology and pathophysiology of IUGR and its treatment.

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