# Proplatelet formation of megakaryocytes is triggered by autocrine-synthesized estradiol

Yuka Nagata,[1,2,5] Jun Yoshikawa,[2]
Atsushi Hashimoto,[1,2] Masayuki Yamamoto,[3]
Anita H. Payne,[4] and Kazuo Todokoro[2,5]

[1]Recognition and Formation, Precursory Research for Embryonic Science and Technology (PRESTO), Japan Science and Technology Corporation (JST), Japan; [2]Cell Fate Signaling Research Unit, RIKEN (The Institute of Physical and Chemical Research), 2-1 Hirosawa, Wako, Saitama 351-0198, Japan; [3]Center for Tsukuba Advanced Research Alliance, University of Tsukuba, Tsukuba 305-8577, Japan; [4]Division of Reproductive Biology, Department of Obstetrics and Gynecology, Stanford University School of Medicine, Stanford, California 94305-5317, USA

A matured megakaryocyte releases thousands of platelets through a drastic morphological change, proplatelet formation (PPF). The megakaryocyte/erythrocyte-specific transcription factor, p45 NF-E2, is essential for initiating PPF, but the factor regulating PPF has not been identified. Here we report that estradiol synthesized in megakaryocytes triggers PPF. We demonstrate that a key enzyme for steroid hormone biosynthesis, 3β-hydroxysteroid dehydrogenase (3β-HSD), is a target of p45 NF-E2, and rescues PPF of p45 NF-E2-deficient megakaryocytes. We also show that estradiol is synthesized within megakaryocytes, and that extracellular estradiol stimulates PPF, inhibition of 3β-HSD activity blocks PPF, and estrogen receptor antagonists inhibit platelet production in vivo. We conclude that autocrine estradiol action regulates platelet production by triggering PPF.

Platelets are produced from pluripotent hematopoietic stem cells in bone marrow through a unique differentiation process (Kaushansky 1995). Thrombopoietin (TPO) stimulates the differentiation of stem cells to megakaryocytes by polyploidization and cytoplasmic maturation. At the last stage of megakaryocyte differentiation, matured polyploid (16N–128N) megakaryocytes produce several lengthy beaded cytoplasmic processes, and this drastic morphological change, called proplatelet formation (PPF), results in the release of thousands of platelets at once (Radley and Scurfield 1980; Shivdasani et al. 1995; Nagahisa et al. 1996; Italiano et al. 1999; Lecine et al. 2000; Rojnuckarin and Kaushansky 2001). It has been

shown that TPO is not required for PPF (Gurney et al. 1994; Shivdasani and Orkin 1995; Shivdasani et al. 1995, 1997; Nagahisa et al. 1996; Bunting et al. 1997; Rojnuckarin and Kaushansky 2001; Oda et al. 2003), and thus the identity of another, as yet unidentified humoral factor that regulates PPF was sought. The search for this PPF-stimulating factor or shedding factor has been unsuccessful to date; thus, the mechanism of PPF and platelet release remains unresolved.

As described below, several lines of evidence suggested that an unidentified intracellular factor, whose expression is up-regulated by the megakaryocyte/erythrocyte-specific transcription factor p45 NF-E2, controls PPF. The identification of this key factor has been sought for a long time. *p45 NF-E2* is expressed mainly in megakaryocyte and erythroid lineage. Although *p45 NF-E2*-deficient (*p45$^{-/-}$*) mice displayed only mild red blood cell abnormality (Shivdasani and Orkin 1995), *p45$^{-/-}$* mice produced megakaryocytes with premature cytoplasm from which platelets could not be released, and thus lacked circulating platelets and died of hemorrhage (Shivdasani et al. 1995, 1997). In TPO receptor-deficient (*c-Mpl$^{-/-}$*) mice, the number of platelets as well as megakaryocytes was decreased (Gurney et al. 1994; Alexander et al. 1996). The megakaryocytes and platelets remaining in *c-Mpl$^{-/-}$* mice, however, were functionally normal, and these mice did not exhibit severe bleeding abnormalities (Bunting et al. 1997). In addition, a delay between TPO injection and peak circulating platelet number has been observed in vivo (Farese et al. 1995). These observations strongly suggested that TPO is not required for PPF, but that the expression of another as yet unidentified factor is up-regulated by p45 NF-E2 in megakaryocytes, and it is this factor that triggers PPF. We therefore attempted the identification of this key factor.

## Results and Discussion

### Establishment of p45$^{-/-}$ and p45$^{+/+}$ ES cells and defect of PPF in p45$^{-/-}$ megakaryocytes

To identify the target genes of p45 NF-E2 and to study their roles in PPF, it was essential to establish *p45$^{-/-}$* ES cells, because most *p45$^{-/-}$* mice die soon after birth and the required number of megakaryocytes is not available for experimentation. The genotypes of established *p45$^{-/-}$* ES cells as well as *p45$^{+/+}$* ES cells were confirmed by PCR analysis (Fig. 1A). In the presence of TPO, *p45$^{-/-}$* ES cells on OP9 stromal cells differentiated into polyploid megakaryocytes but did not display PPF (Fig. 1B, left panel), whereas the majority of *p45$^{+/+}$* ES-derived megakaryocytes (*p45$^{+/+}$* Meg) did exhibit PPF (Fig. 1B, right panel). These data are consistent with in vivo results using *p45$^{-/-}$* mice (Shivdasani et al. 1995).

### 3β-HSD is expressed only in p45$^{+/+}$ Meg

By suppressive subtractive hybridization of cDNAs from *p45$^{-/-}$* ES-derived megakaryocytes (*p45$^{-/-}$* Meg) and those from *p45$^{+/+}$* Meg, the cDNA fragments encoding 3β-hydroxysteroid dehydrogenase (3β-HSD), which is a key regulatory enzyme of all steroid hormone biosynthesis (Abbaszade et al. 1997; Peng et al. 2002), were most
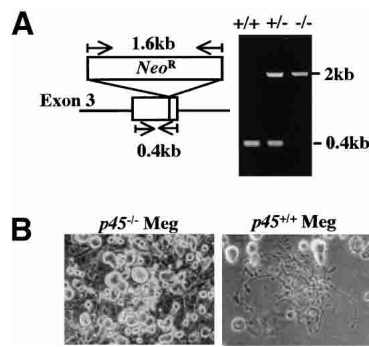
**Figure 1.** Generation of $p45^{-/-}$ and $p45^{+/+}$ ES cells and PPF assay. (*A*) Genomic PCR of established $p45^{-/-}$ and $p45^{+/+}$ ES cells. Map of exon 3 region of mouse *p45 NF-E2* genomic DNA and the locations of the primers used for PCR (*left* panel). Agarose gel electrophoresis of PCR products of $p45^{+/+}$, $p45^{+/-}$, and $p45^{-/-}$ ES cells as indicated (*right* panel). The primers used are 5′-GTTAACTTGCCGGTAG-ATGACTTT-3′ and 5′-AGACCAGCTCAATCTGTAGCCTCC-3′. (*B*) PPF assay of $p45^{-/-}$ Meg and $p45^{+/+}$ Meg. $p45^{-/-}$ and $p45^{+/+}$ ES cells were cocultured on OP9 stromal cells with TPO. (*Left*) $p45^{-/-}$ Meg, from which no proplatelet could be formed. (*Right*) A typical PPF produced from $p45^{+/+}$ Meg.

frequently isolated (~16% among the subtracted genes). The cDNAs of both 3β-HSD I and 3β-HSD VI were isolated, but the type VI isoform cDNA was the major isoform detected in a ratio of 15 to 1. To date, only two genes have been identified as targets of p45 NF-E2 in megakaryocytes (Deveaux et al. 1997; Lecine et al. 2000). One of these is a hematopoietic-specific *β1-tubulin* gene (Lecine et al. 2000), which was the second most abundant gene (5% of the subtracted genes) among the isolated cDNA in our subtraction system. The other is megakaryocyte/platelet differentiation marker, *thromboxane synthase* gene (Deveaux et al. 1997), which was less abundant among the cDNAs isolated. Several other putative p45 NF-E2 target genes were also isolated. The *3β-HSD* gene is, therefore, the third gene that was identified as a p45 NF-E2 target.

Northern blot analysis clearly showed that 3β-HSD transcripts were abundant in $p45^{+/+}$ Meg (Fig. 2A, lane 1), but the transcripts were completely absent in $p45^{-/-}$ Meg (Fig. 2A, lane 2). The transcripts of type I and VI isoforms are not distinguishable by Northern blot analysis. The use of RT-PCR followed by specific restriction enzyme digestion (Abbaszade et al. 1997) demonstrated that both type I and VI isoforms were expressed in $p45^{+/+}$ Meg (Fig. 2B, left panel), whereas only the type I isoform was found in bone marrow megakaryocytes (Fig. 2B, right panel).

To confirm 3β-HSD expression, we prepared anti-3β-HSD I- as well as anti-3β-HSD VI-specific antibodies. The immunoblot analysis with these specific antibodies clearly demonstrated that neither type I nor type VI isoform was present in $p45^{-/-}$ Meg (Fig. 2C, lanes 5), but both isoforms were expressed in $p45^{+/+}$ Meg (Fig. 2C, lanes 4). In undifferentiated $p45^{+/+}$ ES cells, however, neither isoform was detected (Fig. 2C, lanes 3), confirming that 3β-HSD is one of the p45 NF-E2-inducible gene products during megakaryocyte differentiation. Only the type I isoform was expressed in bone marrow megakaryocytes (data not shown). Testis extracts were used as controls (Fig. 2C, lanes 2), because it has been shown that both isoforms are expressed in testes (Baker et al. 1999). These data demonstrated that 3β-HSD is ex-

pressed only in PPF-producing megakaryocytes. We therefore proceeded to study further the role of 3β-HSD in PPF.

### Expression of 3β-HSD *VI gene rescues PPF of* p45$^{-/-}$ *Meg*

The full-length *3β-HSD* VI cDNA was transfected into $p45^{-/-}$ ES cells, and the transfectants were cloned by a limiting dilution. Immunoblot analysis confirmed that the cloned transfectants strongly expressed 3β-HSD VI (Fig. 2C, right panel, lane 6). Transfection of $p45^{-/-}$ ES cells with the *3β-HSD* VI-expressing plasmid rescued PPF of $p45^{-/-}$ Meg (Fig. 3C), whereas the megakaryocytes derived from mock-transfected $p45^{-/-}$ ES cells did not display PPF (Fig. 3A,B). The $p45^{-/-}$ Meg exhibited two types of morphology (Fig. 3A,B), but neither type of megakaryocyte extended the cytoplasmic processes. The majority of $p45^{-/-}$ Meg expressing 3β-HSD VI did exhibit PPF, indicating that $p45^{-/-}$ Meg were induced to synthesize a steroid hormone through the expression of 3β-HSD VI and thus could regulate PPF by an autocrine
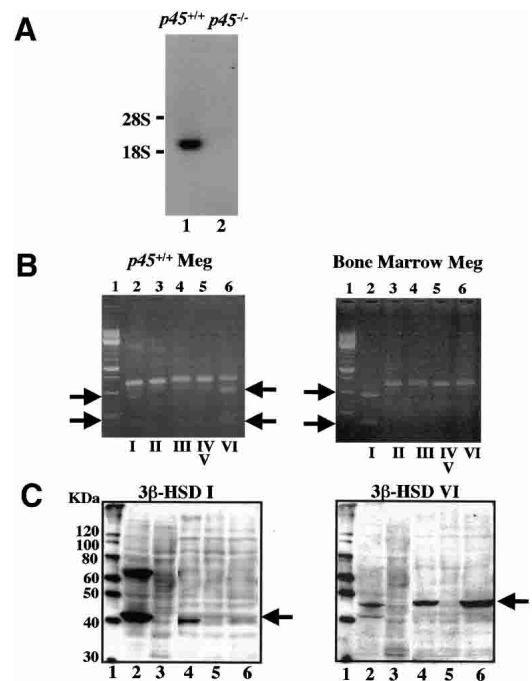


**Figure 2.** Expression of 3β-HSD in $p45^{+/+}$ Meg and bone marrow megakaryocytes but not in $p45^{-/-}$ Meg. (*A*) Expression of 3β-HSD transcripts in $p45^{+/+}$ Meg but not $p45^{-/-}$ Meg. Northern blot analysis was performed with *3β-HSD* cDNA as a probe. (Lane *1*) $p45^{+/+}$ Meg; (lane *2*) $p45^{-/-}$ Meg. (*B*) Expression of 3β-HSD I and/or VI transcripts in $p45^{+/+}$ Meg and bone marrow megakaryocytes. RT-PCR products of $p45^{+/+}$ Meg (*left* panel) and bone marrow megakaryocytes (*right* panel) were digested with unique restriction enzymes, and the isoform types of 3β-HSD transcripts were determined. Roman numerals below indicate 3β-HSD isoform types. (Lane *1*) Molecular weight marker. Arrows indicate the digested cDNA fragments, showing the presence of the designated isoform cDNA in PCR products. (*C*) Immunoblot analysis with 3β-HSD I- and VI-specific antibodies. Total cell extracts prepared from testis (lanes *2*), $p45^{+/+}$ undifferentiated ES cells (lanes *3*), $p45^{+/+}$ Meg (lanes *4*), $p45^{-/-}$ Meg (lanes *5*), and $p45^{-/-}$ Meg expressing 3β-HSD VI (lanes *6*) were probed with anti-3β-HSD I-specific antibody (*left* panel). The same filter was reprobed with anti-3β-HSD VI-specific antibody (*right* panel). (Lane *1*) Molecular weight marker.
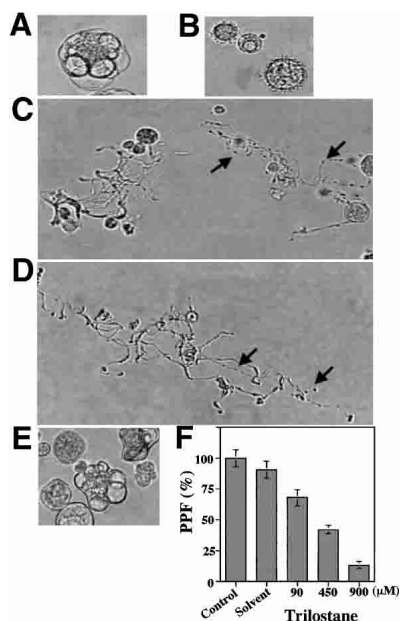
**Figure 3.** Rescue of PPF ability by forced expression of 3β-HSD in *p45*⁻/⁻ Meg and PPF inhibition by trilostane. Cells are shown at the same scale. *p45*⁻/⁻ ES cells were transfected with *3β-HSD* VI cDNA and/or β*1-tubulin* cDNA in expression vectors. The cloned transfectants were cocultured with OP9 cells with TPO, and megakaryocytes producing PPF were observed. (*A,B*) Mock-transfected *p45*⁻/⁻ Meg, which cannot display PPF. (*C*) Two representative proplatelets formed from 3β-HSD VI-expressing *p45*⁻/⁻ Meg. Arrows indicate beaded cytoplasmic processes. (*D*) A typical PPF of *p45*⁻/⁻ Meg expressing both 3β-HSD VI and β1-tubulin. Arrows show long beaded cytoplasmic processes. (*E*) Trilostane-treated bone marrow megakaryocytes. (*F*) Dose-dependent inhibition of PPF by 3β-HSD-specific inhibitor, trilostane. Various concentrations of trilostane dissolved in DMSO were added to bone marrow megakaryocytes, and the number of PPFs formed was counted. Representative data (expressed as mean ± SEM of triplicate determination) from five independent experiments are shown.

mechanism. These data provide evidence that 3β-HSD is essential for PPF in megakaryocytes.

Figure 3C shows two representative proplatelets formed from 3β-HSD-expressing *p45*⁻/⁻ cells. These proplatelets can clearly be characterized as PPF; however, their cytoplasmic processes were not fully extended. Thus, the number of platelets released seemed insufficient, although beaded cytoplasmic processes ready to release platelets were observed (arrows in Fig. 3C). Forced expression of β1-tubulin in *p45*⁻/⁻ ES cells was incapable of restoring PPF (data not shown). This is consistent with previous results obtained with *p45*⁻/⁻ mouse fetal liver megakaryocytes (Lecine et al. 2000). Therefore, we transfected *3β-HSD* VI and β*1-tubulin* together into *p45*⁻/⁻ ES cells. Figure 3D shows a typical proplatelet formed from *p45*⁻/⁻ Meg expressing both 3β-HSD VI and β1-tubulin. The proplatelets bearing several lengthy beaded cytoplasmic processes were produced as normal megakaryocytes, indicating that expression of 3β-HSD is indispensable for PPF and that, in addition, β1-tubulin is required for completing normal PPF.

### 3β-*HSD inhibitor trilostane blocks PPF*

The effect of trilostane, a known specific inhibitor of 3β-HSD (Potts et al. 1978), on PPF in megakaryocytes

isolated from female and male mouse bone marrow was examined. As shown in Figure 3F, trilostane strongly blocked PPF in a dose-dependent manner, whereas the solvent (DMSO) used for dissolving the inhibitor had only a weak inhibitory effect. No gender difference was observed. Trilostane-treated megakaryocytes exhibited irregular shapes, which had several large round intracellular vesicles (Fig. 3E), similar to *p45*⁻/⁻ Meg cells (Fig. 3A). They did not extend any cytoplasmic processes and with time underwent apoptosis. The above results also support that activity of 3β-HSD in megakaryocytes is a prerequisite for PPF, and that *p45*⁻/⁻ Meg as a result of the induction of 3β-HSD acquire the capacity to synthesize steroid hormones. These observations indicate an autocrine mechanism in megakaryocytes leading to PPF.

### *Autocrine synthesis of estradiol and expression of estrogen receptor* β *in megakaryocytes*

Immunoblot analysis confirmed that the receptors for androgen, estrogen (β type), and glucocorticoid were expressed in both *p45*⁺/⁺ Meg and *p45*⁻/⁻ Meg (Fig. 4A), whereas the receptors for estrogen (α type) and progesterone were not detected (data not shown). The expression of steroid hormone receptors in bone marrow megakaryocytes has been described (Tarantino et al. 1994; Khetawat et al. 2000), although their function was not known.

Furthermore, immunostainings of the cells demonstrated that estradiol and progesterone were actually synthesized in male as well as female mouse bone marrow megakaryocytes (Fig. 4B, lower panels) and in *p45*⁺/⁺ Meg (Fig. 4B, upper panels). Little estradiol and progesterone, however, was detected in *p45*⁻/⁻ Meg (Fig. 4B, middle panels). Testosterone was not detected in any megakaryocytes (data not shown), and glucocorticoid synthesis could not be examined because of the unavailability of the antibody. Enzyme immunoassay revealed that male as well as female mouse bone marrow megakaryocytes and *p45*⁺/⁺ Meg, but not *p45*⁻/⁻ Meg, secreted ~140 ± 10 pg of estradiol per $10^5$ cells into the medium during 44 h, and that little progesterone and no testosterone were detected in the media.

### *Extracellular 17*β*-estradiol but not testosterone, progesterone, or dexamethasone stimulates PPF*

Because biosynthesis of estradiol and expression of its receptor in PPF-producible megakaryocytes was verified, we examined the effect of 17β-estradiol on PPF. Addition of 17β-estradiol to the culture medium of CD41⁺ c-Kit⁺ megakaryocyte progenitors in the presence of TPO demonstrated a clear stimulatory effect on PPF in a dose-dependent manner (Fig. 4C), suggesting that 17β-estradiol activates specific targets required for PPF or inactivates specific inhibitors for PPF within megakaryocytes. As described above, megakaryocytes can synthesize estradiol, and thus it was unexpected that the addition of extracellular 17β-estradiol could stimulate PPF. However, addition of 17β-estradiol did enhance PPF >50%, indicating that extracellular hormone could promote further the signals leading to PPF. In contrast, testosterone, progesterone, or the glucocorticoid analog, dexamethasone, had no effect on PPF (data not shown). Progesterone may be synthesized as an intermediate of estrogen, but its receptor is not expressed in megakaryocytes, so that megakaryocytes must be insensitive to progester-
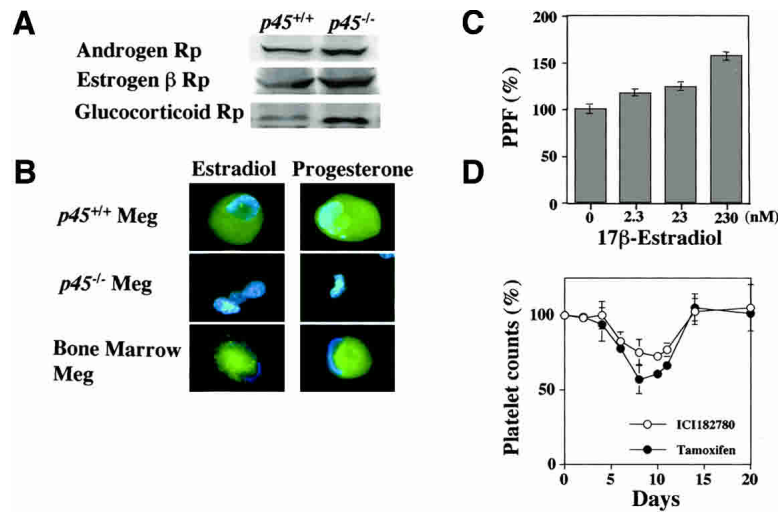
**Figure 4.** Expression of steroid hormones and their receptors in megakaryocytes, PPF stimulation by 17β-estradiol, and estrogen receptor antagonists inhibit platelet production. (*A*) Immunoblot analysis with antisteroid hormone receptor antibodies in megakaryocyte cell extracts. Cell extracts prepared from *p45*$^{-/-}$ Meg (*right* lanes) and *p45*$^{+/+}$ Meg (*left* lanes) were probed with anti-androgen receptor, anti-estrogen receptor β, or anti-glucocorticoid receptor antibody, as indicated. (*B*) Immunostainings of megakaryocytes with anti-steroid hormone antibodies. Bone marrow megakaryocytes (*lower* panel), *p45*$^{-/-}$ Meg (*middle* panel), and *p45*$^{+/+}$ Meg (*upper* panel) were stained with anti-estradiol, anti-testosterone, or anti-progesterone antibody (green) and with DAPI (blue). (*C*) 17β-Estradiol stimulates PPF. Various concentrations of 17β-estradiol were mixed with the culture medium of CD41$^+$ c-Kit$^+$ megakaryocyte progenitors with TPO for 4 d, and PPFs clearly formed were counted. Representative data (expressed as mean ± SEM of triplicate determination) from five independent experiments are shown. (*D*) Estrogen receptor antagonists inhibit platelet production in vivo. Mice were treated with tamoxifen (closed circles) or ICI182780 (open circles) for 9 d, and platelet counts were determined as indicated. The platelet counts in the mice treated with solvent alone (sesame oil containing 5% ethanol) are shown as 100%.

one. Although androgen receptor is expressed, testosterone has no effect on PPF and is not synthesized in megakaryocytes; thus, testosterone is not involved in the process of PPF, as is the case with glucocorticoid, because dexamethasone was found to be without effect.

### Estrogen receptor antagonists inhibit platelet production in vivo

We next examined the effect of estrogen receptor antagonists on platelet production in vivo. ICI182780 or tamoxifen was injected daily for 9 d into mice, and platelet counts were determined in peripheral blood. The result shows that both antagonists inhibited platelet production in vivo (Fig. 4D). The platelet counts in peripheral blood began to decrease 5–6 d after initial injection, decreased further as long as the antagonist was supplied, and reached the lowest level at 8–11 d (Fig. 4D). The maximal decrease in platelet counts observed with ICI182780 and tamoxifen treatment was 25% and 45%, respectively. Following termination of the injection, platelet counts returned to the original level within 5 d. As the half-life of mouse platelets is ~5–6 d, the delay of antagonist effect for 5–6 d suggests that the antagonists inhibited the release of newly produced platelets from the megakaryocytes into peripheral blood. These results demonstrate that estrogen receptor antagonists inhibit platelet production in vivo, and confirm that estradiol stimulates PPF and regulates platelet production.

### Autocrine-synthesized estradiol triggers PPF of mature megakaryocytes

Taken together, these results support the mechanism that 3β-HSD expression is up-regulated by p45 NF-E2 during late stages of megakaryocyte differentiation, which, in turn, induces biosynthesis of estrogen in matured megakaryocytes; finally, autocrine estrogen action triggers the last stage of megakaryocyte maturation, PPF, within megakaryocytes. This sex-independent and differentiation stage-specific autocrine steroid action is an effective mechanism for platelet production, because a small quantity of estradiol can efficiently and rapidly bind to its receptor or other targets and act on PPF within the megakaryocytes in the localized microenvironment of bone marrow.

Our results demonstrate that a sex hormone, estradiol, produced in unexpected tissue hematopoietic cells regulates the last stage of its own cell differentiation by an autocrine mechanism. It was reported that progesterone synthesized in the nervous system stimulates myelin formation and neurite growth (Koenig et al. 1995, 2000). Sex steroids may play important roles in cell differentiation of various unexpected tissues in a sex-independent manner as an autocrine rather than paracrine fashion.

Several aspects of the mechanism of PPF have been described. PKCα plays an important role in PPF by altering actin dynamics (Rojnuckarin and Kaushansky 2001), and β1-tubulin is involved in PPF (Lecine et al. 2000), as also was observed in our study. These results demonstrate that regulatory components of the cytoskeleton are involved in PPF. Furthermore, overexpression of antiapoptotic protein BclxL or Bcl-2 in megakaryocytes reduced PPF (de Botton et al. 2002; Kaluzhny et al. 2002), and PPF was regulated by compartmentalized caspase 3-directed apoptosis (de Botton et al. 2002; Clarke et al. 2003). These results suggest that platelet production is an apoptotic process. Recently, it was suggested that *N*-methyl-D-aspartate (NMDA) receptor-mediated signaling is involved in megakaryocyte maturation and PPF (Hitchcock et al. 2003). Despite these findings, the upstream key factor triggering PPF has not been identified. We report here that estradiol synthesized in megakaryocytes is the factor regulating PPF. As in the case of a "nongenomic" action described in the central nervous system and cardiovascular system (McEwen et al. 2001; Simoncini and Genazzani 2003), estradiol may modulate NMDA receptor as a membrane receptor, transduce signals leading to PPF, and rapidly and effectively respond to the requirement of platelet production. There is now convincing evidence that paracrine-synthesized estradiol can modulate the functions of neural and vascular cells via nongenomic actions (Kelly and Levin 2001). The rapid response of estradiol as well as the rapid inhibition of 3β-HSD indicates a nongenomic action, suggesting that this system may function when an emergency requires a quick platelet production. Alternatively, estradiol may also act by binding to the nuclear receptor and up-regulate specific factors activating PPF or down-

regulate specific inhibitor of PPF as a "genomic" action, as suggested by the detection of estrogen receptor β in megakaryocytes. In this case, a lag time is required until the action occurs, and thus this system may effectively function during nonemergency megakaryocyte differentiation. Both nongenomic and genomic actions may work together or cross-talk with each other in platelet production in vivo. Further studies are required for clarifying how autocrine steroid action correlates with the previous observations described above, and which gene products are up-regulated or down-regulated by estrogen receptor β in megakaryocytes as a genomic action.

### Potential clinical applications of estradiol and 3β-HSD inhibitor/activator

Platelet transfusions are presently the only established therapy for preventing bleeding complications in severely thrombocytopenic individuals such as patients with aplastic anemia or cancer patients on chemotherapy. Furthermore, steroid therapy has been empirically applied for the treatment of various hematopoietic disorders, although its working mechanism was unknown (Negrev 1990; Ranganath et al. 1996; Bord et al. 2000). Our findings suggest that 17β-estradiol or an activator of 3β-HSD, in combination with a low dose of TPO, is effective for the treatment of thrombocytopenia. Furthermore, there is no useful drug for the treatments of thrombocythemia and thrombocytosis. Our data suggest that inhibitors of 3β-HSD such as trilostane and estrogen receptor antagonists may be applicable for the treatments of these diseases.

## Materials and methods

*p45⁻/⁻ and p45⁺/⁺ ES cells and megakaryocyte differentiation*
The $p45^{-/-}$ and $p45^{+/+}$ ES cells were established from the blastocysts of $p45^{+/-}$ mice (Jackson Laboratory), and their genotypes were determined by genomic PCR. The pcDNA3.1-3β-*HSD* VI was transfected into ES cells by electroporation, and 3β-HSD VI expressing transfectants were cloned. The $p45^{-/-}$, $p45^{+/+}$ ES cells or transfectants were cocultured with OP9 stromal cells for 3 d in α-MEM containing 20% fetal calf serum (FCS), and cocultured for a further 8 d in the presence of recombinant mouse TPO (50 units/mL) with medium changes. Recombinant mouse TPO was prepared as described (Nagata et al. 1995).

*Isolation of 3β-HSD cDNA*
Total RNAs were isolated from $p45^{-/-}$ Meg and $p45^{+/+}$ Meg using an RNeasy Mini Kit (QIAGEN). The PCR-Select cDNA subtraction kit (Clontech) was used for cDNA synthesis and suppressive subtractive hybridization, according to the recommended protocol. The cDNA of $p45^{+/+}$ Meg was used as the tester sample, and that of $p45^{-/-}$ Meg as the driver sample. The full-length 3β-*HSD* VI cDNA was isolated and inserted into a pcDNA3.1 expression vector. The 3β-*HSD* VI sequence, in which several nucleotides were different from one in the database, was deposited in the database (accession number AB109387). Northern blot analysis was performed as described (Nagata et al. 2001). The nucleotides 877–1126 of 3β-*HSD* cDNA was used as a probe.

*Determination of isoform type of 3β-HSD transcripts*
Isoform types of 3β-HSD transcripts were determined by RT-PCR followed by restriction enzyme digestion procedure as described (Abbaszade et al. 1997). The primers used were 5′-CAGACCATCCTAGATGT-3′ and 5′-AGGAAGCTCACAGTTTCCA-3′. The RT-PCR products were digested at the unique restriction site of each isoform-specific sequence and separated by 2% agarose gel electrophoresis.

*Preparation of megakaryocytes and PPF assay*
Megakaryocytes from BDF1 mice (6–8-week-old females and males) bone marrow were purified by a modified two-step separation technique as described (Nagahisa et al. 1996). The megakaryocytes ($3 \times 10^3$ cells/mL) were incubated in serum-free medium (S-clone; Sanko) with 1% BSA with or without trilostane (Mochida Pharmaceuticals) at 37°C for 24 h. The CD41⁺ c-Kit⁺ cells ($2 \times 10^3$ cells/mL) prepared as described (Oda et al. 2003) were cultured in S-clone medium with 1% FCS and mouse TPO (50 units/mL) with or without steroid hormones at 37°C for 4 d. Megakaryocytes displaying clear, long cytoplasmic processes were counted.

*Antibody preparation and immunoblot analysis*
Polyclonal anti-3β-HSD I and anti-3β-HSD VI rabbit antisera were prepared by injecting the N-terminal fragment (amino acids 1–267) of 3β-HSD I and C-terminal peptide (amino acids 360–374) of 3β-HSD VI conjugated to KLH, respectively, and 3β-HSD VI-specific antibody was purified by antigen affinity chromatography. Immunoblot analysis was performed as described (Nagata et al. 1995). The cell extracts (75 µg) and testis extracts (50 µg) were applied. Antibodies against 3β-HSD I (1:10000 dilution) and 3β-HSD VI (1:100 dilution), and antibodies against steroid receptors (1:200 dilution; Santa Cruz Biotech) were used. Peroxidase-conjugated AffiniPure F(ab′)₂ fragment donkey anti-rabbit IgG (1:20000 dilution; Jackson ImmunoResearch) was used as a secondary antibody.

*Immunohistochemical analysis*
Immunofluorescence microscopic analysis was performed as described (Nagata et al. 1997), except for the following points. Smear samples of megakaryocytes were fixed with 3.7% formaldehyde at room temperature for 15 min. Anti-estradiol antibody (1:20 dilution; Chemicon), anti-progesterone antibody (1:20 dilution; Chemicon), anti-testosterone antibody (Biogenesis), and Alexa Fluor488 goat anti-rabbit IgG F(ab′)₂ fragment (1:600 dilution; Molecular Probes) were used.

*Enzyme immunoassay of steroid hormones*
An enzyme immunoassay kit (PANTEX) was used to measure quantitatively steroid hormones in the supernatants of megakaryocytes cultured in serum-free medium.

*Platelet counts*
For platelet counts, 100 µL of tamoxifen (2.5 mg/mL) or ICI182780 (2.5 mg/mL) dissolved in solvent (sesame oil:ethanol, 19:1), or solvent alone was injected into BDF1 mice (8-week-old males; *n* = 7) daily for 9 d, and the number of platelets in peripheral blood were counted.

## References

Abbaszade, I.G., Arensburg, J., Park, C.H., Kasa-Vubu, J.Z., Orly, J., and Payne, A.H. 1997. Isolation of a new mouse 3β-hydroxysteroid dehydrogenase isoform, 3β-HSD VI, expressed during early pregnancy. *Endocrinology* **138:** 1392–1399.

Alexander, W.S., Roberts, A.W., Nicola, N.A., Li, R., and Metcalf, D. 1996. Deficiencies in progenitor cells of multiple hematopoietic lineages and defective megakaryocytopoiesis in mice lacking the thrombopoietic receptor c-Mpl. *Blood* **87:** 2162–2170.

Baker, P.J., Sha, J.A., McBride, M.W., Peng L., Payne, A.H., and O'Shaughnessy, P.J. 1999. Expression of 3β-hydroxysteroid dehydrogenase type I and type VI isoforms in the mouse testis during development. *Eur. J. Biochem.* **260:** 911–917.

Bord, S., Vedi, S., Beavan, S.R., Horner, A., and Compston, J.E. 2000. Megakaryocyte population in human bone marrow increases with estrogen treatment: A role in bone remodeling? *Bone* **27:** 397–401.

Bunting, S., Widmer, R., Lipari, T., Rangell, L., Steinmetz, H., Carver-Moore, K., Moore, M.W., Keller, G.A., and de Sauvage, F.J. 1997. Normal platelets and megakaryocytes are produced in vivo in the absence of thrombopoietin. *Blood* **90:** 3423–3429.

Clarke, M.C., Savill, J., Jones, D.B., Noble, B.S., and Brown, S.B. 2003. Compartmentalized megakaryocyte death generates functional platelets committed to caspase-independent death. *J. Cell Biol.* **160:** 577–587.

de Botton, S., Sabri, S., Daugas, E., Zermati, Y., Guidotti, J.E., Hermine, O., Kroemer, G., Vainchenker, W., and Debili, N. 2002. Platelet formation is the consequence of caspase activation within megakaryocytes. *Blood* **100:** 1310–1317.

Deveaux, S., Cohen-Kaminsky, S., Shivdasani, R.A., Andrews, N.C., Filipe, A., Kuzniak, I., Orkin, S.H., Romeo, P.H., and Mignotte, V. 1997. p45 NF-E2 regulates expression of thromboxane synthase in megakaryocytes. *EMBO J.* **16:** 5654–5661.

Farese, A.M., Hunt, P., Boone, T., and MacVittie, T.J. 1995. Recombinant human megakaryocyte growth and development factor stimulates thrombocytopoiesis in normal nonhuman primates. *Blood* **86:** 54–59.

Gurney, A.L., Carver-Moore, K., de Sauvage, F.J., and Moore, M.W. 1994. Thrombocytopenia in c-mpl-deficient mice. *Science* **265:** 1445–1447.

Hitchcock, I.S., Skerry, T.M., Howard, M.R., and Genever, P.G. 2003. NMDA receptor-mediated regulation of human megakaryocytopoiesis. *Blood* **102:** 1254–1259.

Italiano Jr., J.E., Lecine, P., Shivdasani, R.A., and Hartwig, J.H. 1999. Blood platelets are assembled principally at the ends of proplatelet processes produced by differentiated megakaryocytes. *J. Cell Biol.* **147:** 1299–1312.

Kaluzhny, Y., Yu, G., Sun, S., Toselli, P.A., Nieswandt, B., Jackson, C.W., and Ravid, K. 2002. BclxL overexpression in megakaryocytes leads to impaired platelet fragmentation. *Blood* **100:** 1670–1678.

Kaushansky, K. 1995. Thrombopoietin: The primary regulator of platelet production. *Blood* **86:** 419–431.

Kelly, M.J. and Levin, E.R. 2001. Rapid actions of plasma membrane estrogen receptors. *Trends Endocrinol. Metab.* **12:** 152–156.

Khetawat, G., Faraday, N., Nealen, M.L., Vijayan, K.V., Bolton, E., Noga, S.J., and Bray, P.F. 2000. Human megakaryocytes and platelets contain the estrogen receptor β and androgen receptor (AR): Testosterone regulates AR expression. *Blood* **95:** 2289–2296.

Koenig, H.L., Schumacher, M., Ferzaz, B., Thi, A.N., Ressouches, A., Guennoun, R., Jung-Testas, I., Robel, P., Akwa, Y., and Baulieu, E.E. 1995. Progesterone synthesis and myelin formation by Schwann cells. *Science* **268:** 1500–1503.

Koenig, H.L., Gong, W.H., and Pelissier, P. 2000. Role of progesterone in peripheral nerve repair. *Rev. Reprod.* **5:** 189–199.

Lecine, P., Italiano Jr., J.E., Kim, S.W., Villeval, J.L., and Shivdasani, R.A. 2000. Hematopoietic-specific β1 tubulin participates in a pathway of platelet biogenesis dependent on the transcription factor NF-E2. *Blood* **96:** 1366–1373.

McEwen, B., Akama, K., Alves, S., Brake, W.G., Bulloch, K., Lee, S., Li, C., Yuen, G., and Milner, T.A. 2001. Tracking the estrogen receptor in neurons: Implications for estrogen-induced synapse formation. *Proc. Natl. Acad. Sci.* **98:** 7093–7100.

Nagahisa, H., Nagata, Y., Ohnuki, T., Osada, M., Nagasawa, T., Abe, T., and Todokoro, K. 1996. Bone marrow stromal cells produce thrombopoietin and stimulate megakaryocyte growth and maturation but suppress proplatelet formation. *Blood* **87:** 1309–1316.

Nagata, Y., Nagahisa, H., Aida, Y., Okutomi, K., Nagasawa, T., and Todokoro, K. 1995. Thrombopoietin induces megakaryocyte differentiation in hematopoietic progenitor FDC-P2 cells. *J. Biol. Chem.* **270:** 19673–19675.

Nagata, Y., Muro, Y., and Todokoro, K. 1997. Thrombopoietin-induced polyploidization of bone marrow megakaryocytes is due to a unique regulatory mechanism in late mitosis. *J. Cell Biol.* **139:** 449–457.

Nagata, Y., Oda, M., Nakata, H., Shozaki, Y., Kozasa, T., and Todokoro, K. 2001. A novel regulator of G-protein signaling bearing GAP activity for Gαi and Gαq in megakaryocytes. *Blood* **97:** 3051–3060.

Negrev, N. 1990. Female sex hormones and thrombocytopoiesis. *Eksp. Med. Morfol.* **29:** 57–62.

Oda, M., Kurasawa, Y., Todokoro, K., and Nagata, Y. 2003. Thrombopoietin-induced CXC chemokines, NAP-2 and PF-4, suppress polyploidization and proplatelet formation during megakaryocyte maturation. *Genes Cells* **8:** 9–16.

Peng, L., Arensburg, J., Orly, J., and Payne, A.H. 2002. The murine 3β-hydroxysteroid dehydrogenase (3β-HSD) gene family: A postulated role for 3β-HSD VI during early pregnancy. *Mol. Cell. Endocrinol.* **187:** 213–221.

Potts, G.O., Creange, J.E., Hardoing, H.R., and Schane, H.P. 1978. Trilostane, an orally active inhibitor of steroid biosynthesis. *Steroids* **32:** 257–267.

Radley, J.M. and Scurfield, G. 1980. The mechanism of platelet release. *Blood* **56:** 996–999.

Ranganath, L.R., Christofides, J., and Semple, M.J. 1996. Increased mean platelet volume after oestrogen replacement therapy. *Ann. Clin. Biochem.* **33:** 555–560.

Rojnuckarin, P. and Kaushansky, K. 2001. Actin reorganization and proplatelet formation in murine megakaryocytes: The role of protein kinase Cα. *Blood* **97:** 154–161.

Shivdasani, R.A. and Orkin, S.H. 1995. Erythropoiesis and globin gene expression in mice lacking the transcription factor NF-E2. *Proc. Natl. Acad. Sci.* **92:** 8690–8694.

Shivdasani, R.A., Rosenblatt, M.F., Zucker-Franklin, D., Jackson, C.W., Hunt, P., Saris, C.J., and Orkin, S.H. 1995. Transcription factor NF-E2 is required for platelet formation independent of the actions of thrombopoietin/MGDF in megakaryocyte development. *Cell* **81:** 695–704.

Shivdasani, R.A., Fielder, P., Keller, G.A., Orkin, S.H., and de Sauvage, F.J. 1997. Regulation of the serum concentration of thrombopoietin in thrombocytopenic NF-E2 knockout mice. *Blood* **90:** 1821–1827.

Simoncini, T. and Genazzani, A.R. 2003. Non-genomic actions of sex steroid hormones. *Eur. J. Endocrinol.* **148:** 281–292.

Tarantino, M.D., Kunicki, T.J., and Nugent, D.J. 1994. The estrogen receptor is present in human megakaryocytes. *Ann. NY Acad. Sci.* **714:** 293–296.