—Brief Note—

Establishment of a Small-scale Western Blotting System Named as "Micro-Western Blotting" for Mammalian Ova Analysis

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Abstract: *The purpose of the present study was to try to improve the western blotting method in order to reduce the number of sampling ova for protein detection. We established a small-scale western blotting system, named "micro-western blotting", in which the width of each lane was 1 mm and 2 μl sample was applied to each lane in SDS-PAGE. In this method, only 4 porcine ova were required to detect and analyze the phosphorylation states of $p34^{cdc2}$, a catalytic subunit of maturation promoting factor, and two major components of the MAP kinase cascade, ERK and MEK. The number of ova required for protein detection in this method was about one tenth of that required for the normal-scale method and the resolution qualities were comparable with the normal-scale method. The present system might be useful for analyzing biological molecules in small and rare materials such as mammalian oocytes and embryos.*

Key words: *Western blotting, Porcine oocyte, MPF, MAP kinase, MEK*

Western blotting analysis is one of the most popular and useful methods for analyzing biological molecules at the protein level. Also in mammalian ova, regulatory molecules of oocyte maturation and early embryonic development, such as maturation promoting factor (MPF) and the mitogen-activated protein (MAP) kinase cascade, have had their concentrations and phosphorylation states analyzed using this method in several species [1–9]. In previous reports, about 50 eggs were used in each lane for direct protein detection [3–9], and almost 200 eggs were used after immunoprecipitation for detecting the precipitated molecules by western blotting [3]. The requirement of a high protein amount for its detection is one limitation of the western blotting method for mammalian ova analysis.

The purpose of the present study was to try to improve the western blotting method in order to reduce the number of ova samples required for protein detection. We established a small-scale western blotting system, named "micro-western blotting", and in this method, the number of porcine ova required for protein detection was about one tenth of that of the normal-scale method. Furthermore, only a few ova were required to detect $p34^{cdc2}$, a catalytic subunit of MPF, and two major components of MAP kinase cascade, ERK and MEK.

Oocyte collection and in vitro maturation have been described previously [10]. Briefly, ovaries of prepubertal gilts were collected at a local abattoir and cumulus-oocyte complexes (COCs) with intact cumulus cells were aspirated from follicles (2–5 mm in diameter). Some of them were used without culture after removing the surrounding cumulus cells by pipetting. The remaining COCs were cultured (10–15 COCs/100 μl) in TYH [11], supplemented with 1.0 IU/ml pregnant mare's serum gonadotropin (Peamex; Sankyo Co., Tokyo, Japan) and 20% porcine follicular fluid collected as described previously [10], for 48 or 72 hr at 38°C, 5% CO_2 in air. After culture, the COCs were treated with 150 U hyaluronidase (Type IV-S; Sigma, St. Louis, MO) in TYH for a few minutes and then the surrounding cumulus cells were removed by pipetting. The cumulus-free oocytes were boiled in Laemmli buffer [12] for 5 min at a concentration of 60 oocytes/8 μl and stored at –80°C until use.

In the present study, a small-scale western blotting named the "micro-western blotting (micro-WB)" and a normal-scale western blotting (normal-WB) as a control

Received: July 12, 1999
Accepted: September 7, 1999
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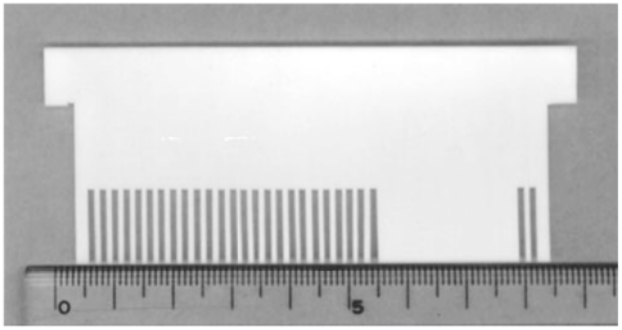

Fig. 1. A comb used for micro-western blotting. A measure is shown for indication of its size.

were performed. The methods were almost the same between these two types of western blotting except for their scale. Running-gel sizes (width × height × thickness) were 135 × 100 × 1 mm and 80 × 50 × 0.5 mm for normal- and micro-WB, respectively. A comb, 1 mm width for each lane and 1 mm width for inter-lane, was ordered for the micro-WB (Fig. 1) and 4 to 15 oocytes per 2 μl were applied using a micro-syringe, whereas the width of each lane in the normal-WB was 5 mm and 4 to 50 oocytes per 20 μl were applied. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed according to the method of Laemmli [12], using a 10% original gel (composed of 360 mM Tris-HCl, 10% acrylamide, 0.27% Bis, 0.1% TEMED, 0.025% ammonium persulfate and 0.1% SDS) for cdc2, and 10% modified gel (composed of 375 mM Tris-HCl, 10% acrylamide, 0.1% Bis, 0.1% TEMED, 0.025% ammonium persulfate and 0.1% SDS) for ERK and MEK. After SDS-PAGE, the proteins were transferred to a polyvinylidiene fluoride membrane (AE-6660; Atto, pore size 0.2 μm) and blocked with 5% skimmed milk in TBS containing 0.1% tween-20. The antibodies used were an anti-ERK polyclonal antibody (sc-94: Santa Cruz Biotechnology), an anti-MEK-1 monoclonal antibody (MD-16-3; MBL) and an anti-p34^{cdc2} (PSTAIRE) monoclonal antibody [13]. Signals were detected using a blotting detection kit (Amersham International, Amersham, Buckinghamshire, UK) according to the manufacturer's instructions. Immunoblotting experiments were repeated at least two times.

The results of western blotting are shown in Fig. 2. When 50 non-cultured porcine oocytes were immunoblotted with anti-ERK, anti-MEK and anti-PSTAIRE antibodies, two specific bands at 44 and 42 kDa, one band at 45 kDa and one band at 34 kDa were detected by normal-WB as reported previously [3–9]. These bands were also detected by micro-WB and the densities of the bands were well correlated with the number of oocytes used from 15 to 4. The width of each band was about 2 mm in micro-WB as shown in bottom panel of Fig. 2A and Fig. 2B, whereas that of the normal-WB was about 6 mm.

These bands have been reported to represent dephosphorylated non-active forms of ERK1, ERK2, MEK1 and cdc2, respectively, and these molecules are phosphorylated in matured oocytes at the sites regulating their activity [2–9]. It has been also reported that the phosphorylated forms of these molecules migrate slowly on SDS-PAGE and are detectable as shifted-up bands by western blotting [2–9]. It is important to clarify whether the phosphorylation forms could be distinguished by micro-WB. When porcine oocytes cultured for 48 hr were immunoblotted by the same antibodies, clear shift-up bands were detected by micro-WB as well as normal-WB, and only 4 oocytes were required to detect these phosphorylated forms in micro-WB. In contrast, only faint bands and no bands were detected when 10 and 4 oocytes were immunoblotted by normal-WB, respectively, and these data might not be available for the analysis.

In the case of p34^{cdc2}, an additional upper band which represents hyperphosphorylated cdc2 has been reported in mitotic cells, although the band is very weak and almost undetectable during oocyte maturation until 48 hr [3]. We found recently that the band density increased gradually by additional culture [14]. Therefore, we next attempted to detect this hyperphosphorylated form by micro-WB using porcine oocytes cultured for 72 hr. The third band was identified by micro-WB using only 5 oocytes as clear as normal-WB using 50 oocytes.

These results show that the present scale down of the western blotting system has almost no detrimental effects on the resolution qualities of the targeted molecules, and indicate that the present small-scale western blotting in which the required amount of materials for protein detection is about one tenth of that required for the normal-scale method is a useful system for analyzing biological molecules in small and rare materials such as mammalian oocytes and embryos.

Aknowledgements

We thank Professor Masakane Yamashita, Hokkaido University, for the generous gift of an anti-PSTAIRE antibody. This work was supported by Grant-in-Aid for Scientific Research (no. 10660267 and no. 11556051 to KN) from the Ministry of Education, Science, Sports and Culture of Japan.

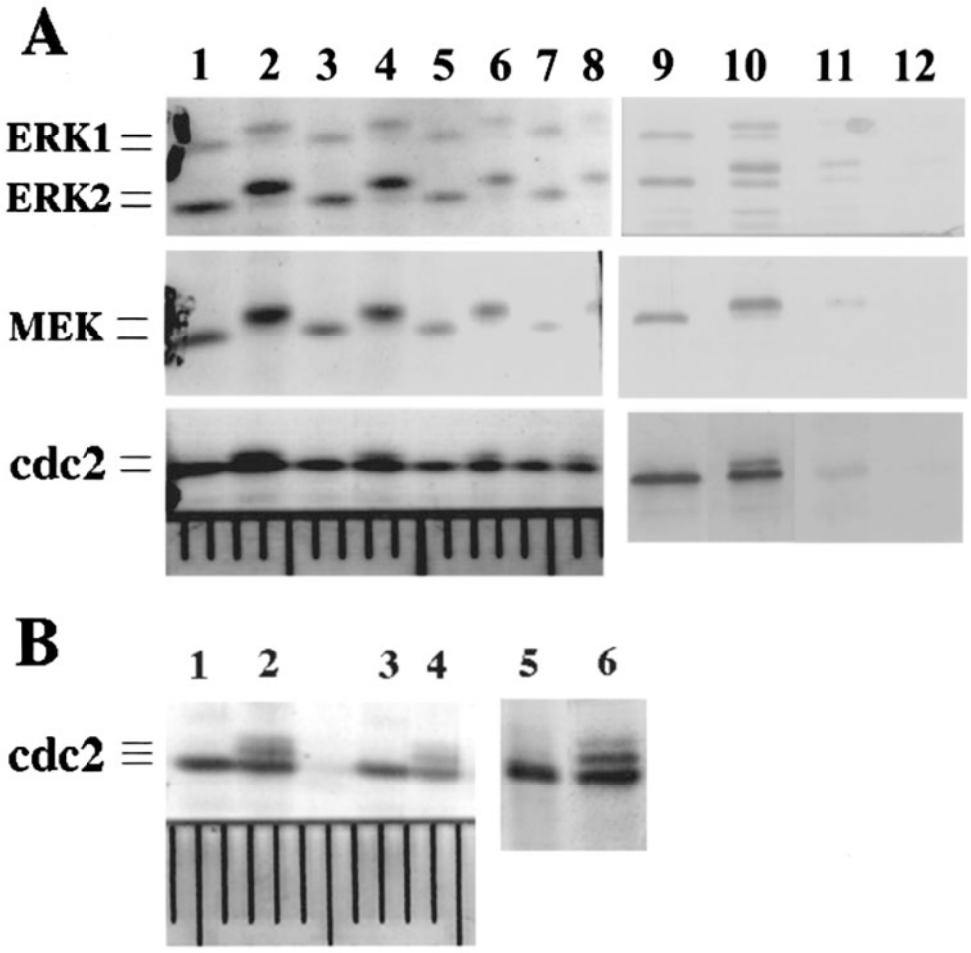

Fig. 2. Detection of ERKs, MEK and $p34^{cdc2}$ by micro-western blotting. A, Porcine oocytes non-cultured (lanes 1, 3, 5, 7 and 9) or cultured for 48 hr (lanes 2, 4, 6, 8, 10, 11 and 12) were subjected to micro- (lanes 1–8) or normal- (lanes 9–12) western blotting with anti-ERKs (top panel), anti-MEK (middle panel) and anti-$p34^{cdc2}$ (bottom panel) antibodies. The numbers of oocytes used were 15 (lanes 1 and 2), 10 (lanes 3, 4 and 11), 6 (lanes 5 and 6), 4 (lanes 7, 8 and 12) and 50 (lanes 9 and 10). The scales shown in the bottom panel lane 1–8 indicate 1 mm for 1 scale. B, Porcine oocytes non-cultured (lanes 1, 3 and 5) or cultured for 72 hr (lanes 2, 4 and 6) were subjected to micro- (lanes 1–4) or normal- (lanes 5 and 6) western blotting with anti-$p34^{cdc2}$ antibodies. The number of used oocytes were 10 (lanes 1 and 2), 5 (lanes 3 and 4) and 50 (lanes 5 and 6). The scales shown in the lanes 1–4 indicate 1 mm for 1 scale.

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