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Surface plasmon resonance-biosensor detects the diversity of responses against epidermal growth factor in various carcinoma cell lines

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Abstract

Surface plasmon resonance (SPR) biosensor detects intracellular signaling events as a change of the angle of resonance (AR). We previously reported that the activation of epidermal growth factor receptor (EGFR) on keratinocytes causes a unique triphasic change of AR, whereas the activation of other receptors, such as IgE receptor and adenosine A3 receptor on mast cells, causes a transient monophasic increase of AR. To study the mechanism of AR changes induced by EGFR activation, we introduced wild and mutated EGFR cDNAs into Chinese hamster ovary (CHO) cells and analyzed changes of AR in response to EGF. CHO cells expressing wild-type EGFR showed a triphasic change of AR, whereas cells expressing kinase-dead EGFR (K721M) showed minimum change of AR. A phosphatidylinositol 3-kinase inhibitor, wortmannin, attenuated the third phase of AR change in CHO cells expressing wild-type EGFR. The pattern of AR change was independent on the concentration of EGF. We also analyzed changes of AR with a nontumorigenic keratinocyte cell line, HaCaT, and several cell lines of carcinoma to explore the feasibility of SPR biosensor as a tool for clinical diagnosis. The activation of HaCaT cells and one out of six carcinoma cell lines showed a full triphasic change of AR. In contrast, five out of the six cell lines showed mono- or bi-phasic change of AR. These results suggest that EGF induces the SPR signals via the

phosphorylation of EGFR, and provide a possibility that the SPR biosensor could be applied to the real-time detection and diagnosis of malignant tumors.

(250 words)

Key words: Biosensor; Surface plasmon resonance; Living cell; Cancer; Epidermal growth factor; Diagnosis

1. Introduction

Surface plasmon resonance (SPR) biosensors are capable of characterizing the binding of detectants in the field of resonance on a sensor chip in real-time without any labeling (Homola, 2003). They are useful to study the interactions of biological molecules from proteins, oligonucleotides, and lipids through to small substances, such as phages, viral particles, and cells (Rich and Myszka, 2000). We previously reported that SPR sensors can detect unexpectedly large changes of the angle of resonance (AR), when adherent RBL-2H3 mast cells (Hide et al., 2002) or non-adherent human basophils (Yanase et al., 2007b) were cultured on a biosensor chip and stimulated by antigen, indicating that SPR biosensors could be appropriate for the real time and non-label detection of the activation of living cells. In addition, we recently found that the change of AR reflects intracellular events in living cells rather than changes in the size of the area to which cells adhere (Yanase et al., 2007a), and that the antigen-induced increase of AR in RBL-2H3 cells are largely depend on the activation of PKCβ (Tanaka et al., 2008). RBL-2H3 cells consistently showed a monophasic increase of AR in response to antigen stimulation (Hide et al., 2002). In contrast, PAM212 keratinocytes, which were stimulated with epidermal growth factor (EGF), typically exhibited a triphasic change of AR (Yanase et al, 2007a). However, the exact

mechanisms, which cause the differences in patterns of AR change with different types of cells or different types of stimulation, remain undetermined.

Epidermal growth factor receptor (EGFR), one of the ErbB family of receptor tyrosine kinases, is a transmembrane tyrosine kinase receptor promoting proliferation and survival of both normal and cancer cells. EGFR dimerization by its ligand, EGF, causes the phosphorylation of several tyrosine residues, and then recruits and activates the subsequent signaling molecules, such as phospholipase Cγ, c-Cbl, p85 subunit of phosphatidylinositol 3-kinase (PI3K), Grb1/2, Shc, and Shp1 (Ono and Kuwano, 2006). Some of the abnormal activation of EGFR in cancer cells is due to an amplification of EGFR, or gain-of-function mutations of EGFR in its tyrosine kinase domain (Normanno et al., 2006). The EGFR-targeted therapies, using small molecule tyrosine kinase inhibitors (erlotinib and gefitinib) or monoclonal antibodies against EGFR (cetuximab and panitumumab), have effectively improved outcomes for patients with colorectal, lung, head and neck, and pancreatic cancers (Laurent-Puig et al., 2009).

In this study, we established exogenous human EGFR-expressing Chinese Hamster Ovary (CHO) cells and studied an EGF-induced change of AR. We also analyzed the change of AR induced by EGF with several human carcinoma cell lines that express EGFR as well as a nontumorigenic human keratinocyte cell line, HaCaT,

and revealed that different types of cells may show different patterns of SPR signals.

2. Materials and methods

2.1. Reagents and antibodies

The recombinant human epidermal growth factor was from R&D systems (Minneapolis, MN). A PI3K inhibitor, wortmannin, was from Sigma-Aldrich (St. Louis, MO). The monoclonal antibody against FLAG was from Stratagene (La Jolla, CA). The polyclonal antibodies against EGFR, phospho-EGFR (pY1148), Akt, and phospho-Akt (pT308) were from Cell Signaling Technology (Beverly, MA).

2.2. Human carcinoma cell lines and a nontumorigenic human keratinocyte cell line, HaCaT

The gastric cancer cell lines, MKN-1, MKN-7, TMK-1, and MKN-28 (Yoshida et al., 1990) were generous gifts from Dr. Yasuhiko Kitadai, Department of Medicine and Molecular Science, Hiroshima University Graduate School of Biomedical Sciences. The prostate cancer cell lines, DU145 and LNCap, were kindly provided from Dr. Hiroaki Yasumoto, Department of Urology, Graduate School of Medical Sciences, Hiroshima University. DU145 cells were maintained in MEM supplemented with 10%

fetal calf serum (FCS) and antibiotics. All other cancer cell lines were maintained in RPMI1640 supplemented with 10% FCS and antibiotics. HaCaT cells (Boukamp et al, 1988) were maintained in DMEM supplemented with 10% fetal calf serum (FCS) and antibiotics.

2.3. EGFR expression vectors

The human EGFR cDNA (pco12 EGFR) was provided by DNA Bank, BioResource Center, RIKEN (Tsukuba, Ibaraki, Japan), and the cDNA was amplified by PCR with the following primer pair, 5'-TGCAGGATATCGCTCTTCGGGGAGCAGCGATG-3' and 5'-CCGCTCGAGTGCTCCAATAAATTCACTGCTTTG-3'. The PCR product was digested with *EcoR V* and *Xho I* and ligated into the same sites of pCMV-Tag4 vector (Stratagene) for the addition of C-terminal FLAG epitope. The EGFR with the mutation on the ATP binding site (K721M) (Chen et al., 1987) was generated by the QuikChange^{R} Site Directed Mutagenesis Kit (Stratagene) and the following primer pair; the sense primer: 5'-GTTAAAATTCCCGTCGCTATCATGGAATTAAGAGAAGCAACATCTC-3', the antisense primer:

5’-GAGATGTTGCTTCTCTTAATTCCATGATAGCGACGGGAATTTTAAC-3’.

2.4. Transfection

CHO-K1 cells (RCB0285) were provided by RIKEN Cell Bank (Tsukuba, Ibaraki, Japan) and maintained with Ham’s F-12 (Invitrogen) containing 10% FCS and antibiotics. Cells were transfected with FLAG-tagged EGFRs and were selected by 1 mg/ml G418 for two weeks. The clones that stably expressed the FLAG-tagged EGFRs were selected by immunoblotting using anti-FLAG antibody.

2.5. Western blot analysis

Cells were washed twice with ice-cold phosphate buffered saline and lysed in the lysis buffer (25 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% nonidet P-40, 5 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 10 mM sodium fluoride, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml leupeptin, 25 μg/ml aprotinin, and 2 μg/ml pepstatin) (Hiragun, et al., 2006) and incubated for 30 min on ice. The cell lysates were separated by SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene fluoride membrane (Immobilon-P; Millipore, Billerica, MA). The membrane blots were probed with the indicated primary antibodies, and the

immunoreactive proteins were visualized by the use of horseradish peroxidase-conjugated secondary antibodies and chemiluminescence.

2.6. Flow cytometric analysis

The CHO cells expressing wild-type EGFR or EGFR-K721M were cultured on RepCell^{TM} plates (CellSeed, Tokyo, Japan), detached at low temperature, and then stained with PE-conjugated anti-EGFR monoclonal antibody (BD Bioscience, Franklin Lakes, NJ) or its isotype control. The surface expression of EGFR was determined with FACSCalibar^{TM} (BD Bioscience).

2.7. SPR analysis

The method measuring the change of AR of living cells in response to antigen stimulation was described previously (Hide et al., 2002; Yanase et al., 2007a). Briefly, on the day before experiments, cells were seeded on biosensor chips (1.2×10^4 cells/60 μl/site) and cultured overnight. The sensor chip was equipped in a flow-cell unit of the SPR apparatus, SPR-CELLIA (Moritex, Nagoya, Japan). The cells placed in the chambers were preincubated for 20 min with glucose saline/Pipes buffer (Hiragun et al., 2006) and stimulated with 10 ng/ml of EGF for 10 min. The change of AR during

cellular activation was monitored by SPR-CELLIA for up to 30 min.

3. Results

3.1. CHO cells expressing the wild type EGFR and HaCaT cells show triphasic changes of AR in response to EGF

CHO cells, which do not possess endogenous EGFR (Chen et al., 1987; Krug et al., 2002), were transfected and the CHO cell clone stably expressing the exogenous wild type human EGFR was established. The mock or EGFR-transfected cells were stimulated with EGF and the change of AR was monitored by the SPR biosensor. As shown in Fig. 1A, EGFR-transfected cells showed a typical triphasic change in response to EGF. The first phase was short, tiny spike like increase in response to EGF. The second phase was the following quick decrease, and the third phase was the relatively slow increase of AR close to the base line. HaCaT is a spontaneously transformed human epithelial cell line from adult skin, and it is immortal but nontumorigenic (Boukamp et al, 1988). HaCaT cells also showed the triphasic change of AR in response to EGF (Fig. 1B).

3.2. CHO cells expressing the EGFR mutated on the ATP-binding domain show the

minimal change of AR in response to EGF

We next studied the change of AR in cells expressing exogenous EGFR mutated on the ATP binding site (K721M). These cells were not phosophorylated in the tyrosine residues of EGFR even in the presence of EGF (Fig. 2A). Equivalent expressions of the wild type and kinase-defect EGFR were confirmed by immunoblot and flow cytometric analyses (Fig. 2A and 2B, respectively). Cells expressing mutated EGFR showed only a minimal change of AR in response to EGF stimulation, when compared with cells expressing wild type EGFR (Fig. 2C).

3.3 A PI-3 kinase inhibitor, wortmannin, attenuates the third phase of AR change induced by EGF

The ligation of EGF and EGFR induces the activation of numerous signaling molecules. We investigated the role of PI3K, one of the important signaling molecules strongly involved in cell proliferation, on the SPR signal induced by EGF. A specific PI3K inhibitor, wortmannin, abolished the activation of Akt, the downstream signaling molecule of PI3K (Fig. 3A). Wortmannin also attenuated the third phase of the SPR signal when compared with vehicle control (dimethyl sulfoxide), suggesting that PI3K specifically involved in that phase of SPR signal (Fig. 3B).

3.4 The pattern of AR change was not dependent on the concentration of EGF

We next investigated whether the pattern of AR change is dependent on the concentration of EGF or not. We stimulated CHO cells, which expressed wild type EGFR, with 0.1, 1, or 10 ng/ml of EGF. Western blot analysis revealed a strong tyrosine phosphorylation of EGFR in the cells stimulated with 10 ng/ml of EGF, a much reduced level of phosphorylation with 1 ng/ml of EGF, and no apparent phosphorylation with 0.1 ng/ml of EGF (Fig. 4A). Thus, we stimulated the cells with 1 or 10 ng/ml of EGF and monitored the change of AR. The cells stimulated with 1 ng/ml of EGF showed a smaller, but the same pattern of AR change as that induced by 10 ng/ml of EGF (Fig. 4B).

3.5. Carcinoma cell lines exhibited diversities in changes of AR induced by EGF

We examined changes of AR in response to EGF with six human cancer cell lines, consisting of four gastric cancer cell lines and two prostate cancer cell lines. The expression and tyrosine phosphorylation of EGFR in response to EGF stimulation were confirmed by western blot analysis with antibodies against EGFR and phosphorylated EGFR (Fig. 5, inserted figures). The degree of increase or decrease of AR with cell lines

was variable among experiments and cell lines. However, patterns of AR changes in each cell line were the same throughout experiments (Fig. 5). Namely, TMK-1 cells showed small, but all three components of AR changes induced by EGF, but MKN-1, MKN-7, and MKN-28 cells showed two, and DU145 and LNCap cells showed only one component of the AR change in response to EGF stimulation. In particular, the reaction pattern of AR with LNCap, a prostate cancer cell line, was largely different from the other five cell lines in that it showed only monophasic descending change of AR in response to the EGFR activation. The sequence analysis of EGFR in LNCap revealed no mutations in its tyrosine kinase domain (data not shown).

4. Discussion

In this study, we demonstrated that a CHO cell clone expressing the wild type human EGFR showed the typical SPR signals consisting of three phases of AR changes in response to EGF stimulation as well as nontumorigenic HaCaT cells. In contrast, another CHO cell clone expressing EGFR with a kinase-dead mutation on K721M exhibited no or only a minimal change of SPR signals. Since EGF did not induce tyrosine phosphorylation in the mutated receptor even though EGF could similarly bind to both wild-type and mutated receptors (Chen et al., 1987), our results confirmed that

EGF-induced SPR signals were not simply evoked by ligand binding, but by tyrosine phosphorylation of EGFR and/or subsequent signaling events of the activated EGFR. Moreover, a PI3K inhibitor, wortmannin, attenuated the third phase of AR change in CHO cells (Fig. 3), indicating that PI-3 kinase involved in the last phase of SPR signal evoked by EGF stimulation. In agreement with these results, we previously reported that genistein almost abolished the whole SPR response, whereas wortmannin suppressed the late phase of the response in RBL-2H3 cells stimulated with antigen (Hide, et al., 2002).

The intensity of SPR signals may vary among the experiments, but the pattern of SPR signals always has good reproducibility. In the present study, we demonstrated that the pattern of SPR response was not dependent on the concentration of EGF, but presumably determined by the type of cell and stimulation. The activation of six different cell lines of human carcinoma with EGF showed diverse signals of SPR not only in the intensity, but also in the pattern of their kinetics. One of four gastric carcinoma cell lines, TMK-1 cells showed small, but all three components of AR changes, but the other gastric carcinoma cell line, MKN-1, MKN-7, and MKN-28 cells showed only two components of AR changes in response to EGF. Furthermore, two prostate carcinoma cell lines DU145 and LNCap cells showed only one component of

AR changes. The reaction pattern of LNCap cells was particularly characteristic in that it largely decreased without a sign of increase. The DNA sequence of EGFR derived from LNCap showed no mutation in its kinase domain, indicating that the characteristic pattern of SPR signals of LNCap was not due to the mutations in EGFR, but subsequent signaling events following the activation of receptors. To clarify the involvement of PI-3 kinase in the pattern of SPR signals of human carcinoma cell lines, we examined the phosphorylation of Akt in response to EGF stimulation in those six carcinoma cell lines. The expression and basal phosphorylation of Akt varied among cell lines, but Akt was further phosphorylated in all cell lines in response to EGF (Supplementary figure 1). Our findings indicate that, to induce the last phase of AR change, the activation of PI-3 kinase is necessary but insufficient by itself, and presumably requires other molecules downstream of the cascade, which is different from Akt phosphorylation. Further investigations on EGFR-mediated molecular actions in LNCap cells should elucidate relations of SPR signal components to molecular events induced by EGFR phosphorylation.

Cancers are characterized by self-sufficiency in growth signals, insensitivity to anti-growth signals, evasion of programmed cell death (apoptosis), limitless replicative potential, sustained angiogenesis, and tissue invasion and metastasis (Hanahan and

Weinberg, 2000). In general, the diagnosis of cancers are made by a histopathological observation of either cellular or tissue level abnormality in their structures, such as cell atypicality and the presence of invasion in the hematoxylin and eosin stained specimens. Immunological staining of the tissue specimens, or recently, genetic analysis of mutations may help the diagnosis. However, all these examinations give mostly morphological, but not functional information regarding tissues and/or cells. Considering features of malignancy in tumors mentioned above, such morphological examinations without functional analysis may be insufficient, or even inappropriate for a diagnosis of cancers and other malignant tumors. Moreover, many of the above mentioned conventional strategies take days or weeks to be completed, and thus might result in a delay of appropriate treatments. In this study, we revealed that LNCap cells exhibit a unique pattern of AR change in response to EGF stimulation. Since the change of AR in individual cells may be observed by two-dimensional SPR-Imaging system (Yanase et al., 2010), the presence of single LNCap cell among normal or even other malignant tumor cells should be detected based on the pattern of SPR signal, as a kinetics of AR in a real-time non-label manner. The accumulation of such information about SPR signals of various malignant tumors could contribute to the improvement of clinical diagnosis of tumors.

5. Conclusion

In this study, we demonstrated that carcinoma cell lines may show various patterns of SPR signals, which are different from those of normal cells. Therefore, the SPR biosensor may be applied to “the functional diagnosis of cancers” by detecting patterns of AR changes without any histological or immunological labeling.

Conflict of interest

The authors declare that they have no conflict of interest to disclose.

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Figure legends

Fig. 1. *CHO cells expressing the wild type EGFR and HaCaT cells show triphasic changes of AR in response to EGF.* (A) Cells transfected with empty vector (mock) or vector coding human wild-type EGFR were seeded onto the sensor chip, cultured overnight, and then stimulated with 10 ng/ml of recombinant human EGF. The change of AR was monitored by SPR-CELLIA for up to 30 min. (B) HaCaT cells were seeded onto the sensor chips, cultured overnight, and then stimulated with 10 ng/ml of EGF. The change of AR was monitored for up to 30 min. A representative data of three independent experiments is shown for each panel.

Fig. 2. *CHO cells expressing the EGFR mutated on the ATP-binding domain show the minimal change of AR in response to EGF.* (A) The cells expressing wild-type or mutated EGFR on K721 were stimulated with 10 ng/ml of EGF for 5 min and then lysed. The whole cell lysates were immunoblotted for the detection of phosphorylated EGFR, total EGFR, or FLAG-tag by the use of the specific antibodies against them. (B) The cells expressing wild type (solid line) or K721M-mutated (dotted line) EGFR were stained with PE-conjugated anti-human EGFR. Black filled histogram indicates the cells expressing wild type EGFR stained with isotype control. (C) The cells expressing wild

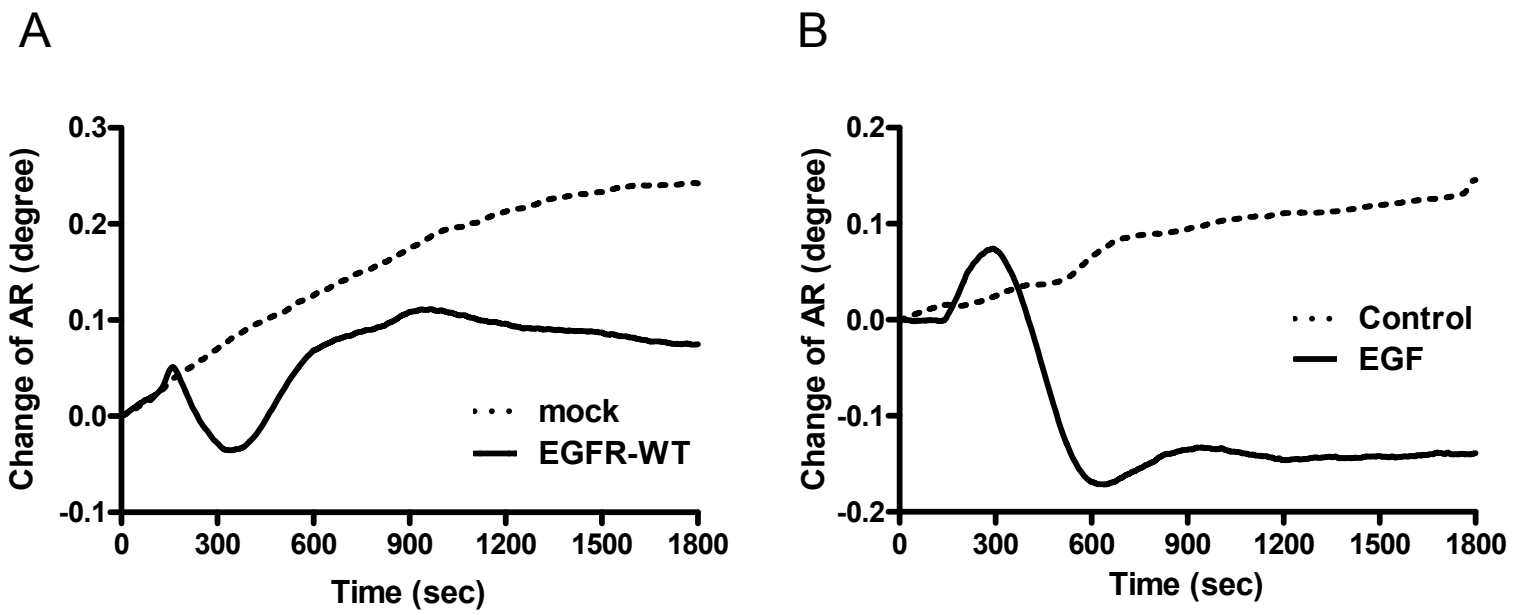
type or K721M mutated EGFR were seeded onto the sensor chips, cultured overnight, and then stimulated with EGF for 10 min. The change of AR was monitored by SPR-CELLIA for up to 30 min. A representative data of three independent experiments is shown for each panel (A-C).

Fig. 3. *A PI3K inhibitor, wortmannin, suppresses the third phase of AR change induced by EGF stimulation.* (A) CHO cells expressing wild type EGFR were pretreated with 100 nM of wortmannin (WMN) or not for 30 min, stimulated with 10 ng/ml of EGF for 5 min, and then lysed. The whole cell lysates were immunoblotted for the detection of phosphorylated Akt or total Akt by the use of the specific antibodies against them. (B) The cells expressing wild type EGFR were seeded onto the sensor chips, cultured overnight, pretreated with vehicle control (Control) or 100 nM of wortmannin (WMN) for 30 min, and then stimulated with 10 ng/ml of EGF for 10 min. The change of AR was monitored by SPR-CELLIA for up to 30 min. A representative data of three independent experiments is shown.

Fig. 4. *Lower concentration of EGF induced the similar pattern of AR change in CHO cells.* (A) CHO cells expressing wild type EGFR were stimulated with 0.1, 1, or 10

ng/ml of EGF for 5 min, and then lysed. The whole cell lysates were immunoblotted for the detection of phosphorylated EGFR or total EGFR by the use of the specific antibodies against them. (B) The cells expressing wild type EGFR were seeded onto the sensor chips, cultured overnight, and then stimulated with 1 or 10 ng/ml of EGF for 10 min. The change of AR was monitored by SPR-CELLIA for up to 30 min. A representative data of three independent experiments is shown.

Fig. 5. *Different carcinoma cell lines exhibited diverse changes of AR in response to EGF.* The gastric cancer cell lines (MKN-1, MKN-7, TMK-1, and MKN-28) and the prostate cancer cell lines (DU145 and LNCap) were seeded onto the sensor chips, cultured overnight, and then stimulated with 10 ng/ml of EGF for 10 min. The change of AR was monitored by SPR-CELLIA for up to 50 min. The inserted figures in each panel showed the tyrosine phosphorylation of EGFR in response to EGF stimulation. TMK-1 cells showed all three components of AR changes induced by EGF, but MKN-1, MKN-7, and MKN-28 cells showed two, and DU145 and LNCap cells showed only one component of the AR changes. A representative data of the indicated number of independent experiments is shown.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0.0
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0
300
600
900
1200
1500
1800
Time (sec)
mock
EGFR-WT
Change of AR (degree)
0.2
0.1
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300
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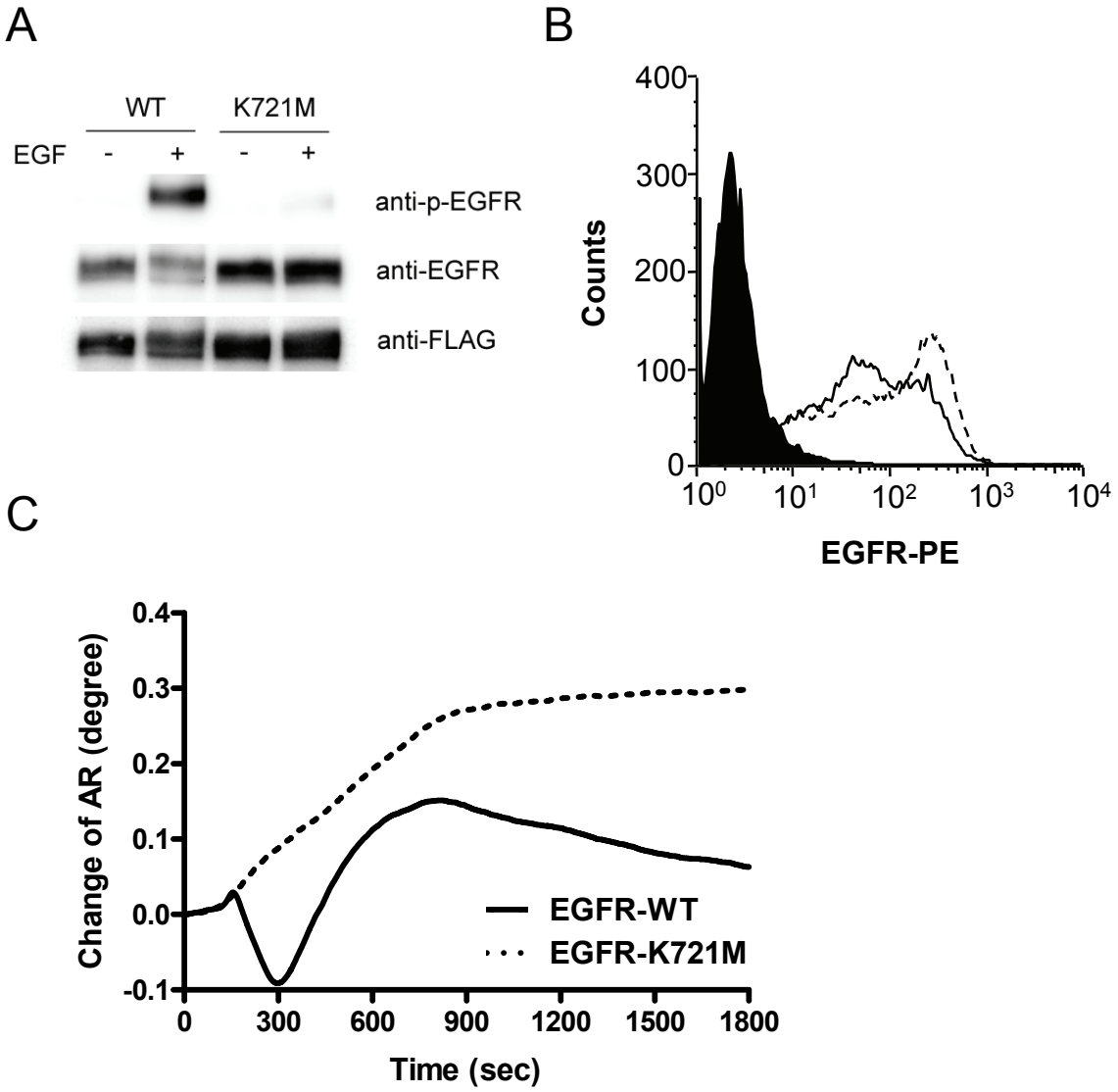
900
1200
1500
1800
Time (sec)
Control
EGF

Fig. 1.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10^3
10^4
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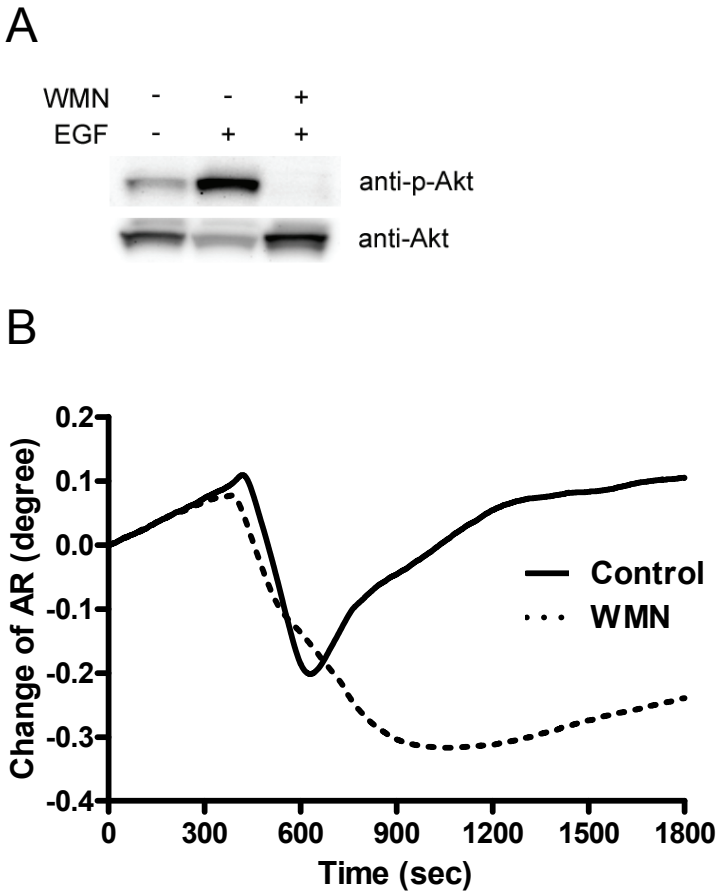
Time (sec)

Fig. 2.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WMN - - +
EGF - + +
anti-p-Akt
anti-Akt
B
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-0.4
0
300
600
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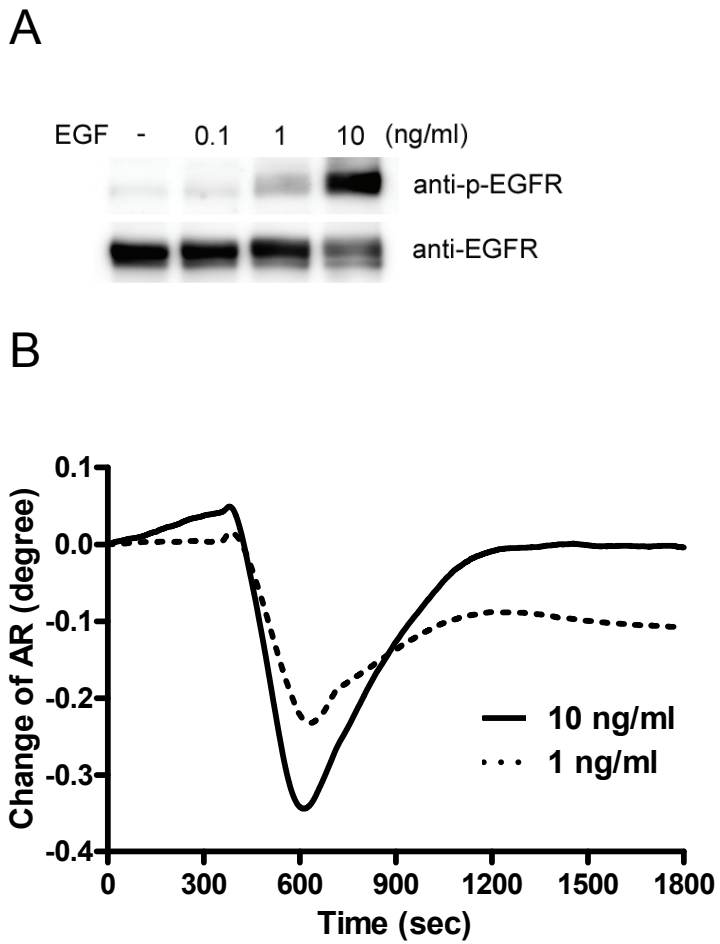
1200
1500
1800
Time (sec)
Control
WMN

Fig. 3.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anti-p-EGFR
anti-EGFR
B
Change of AR (degree)
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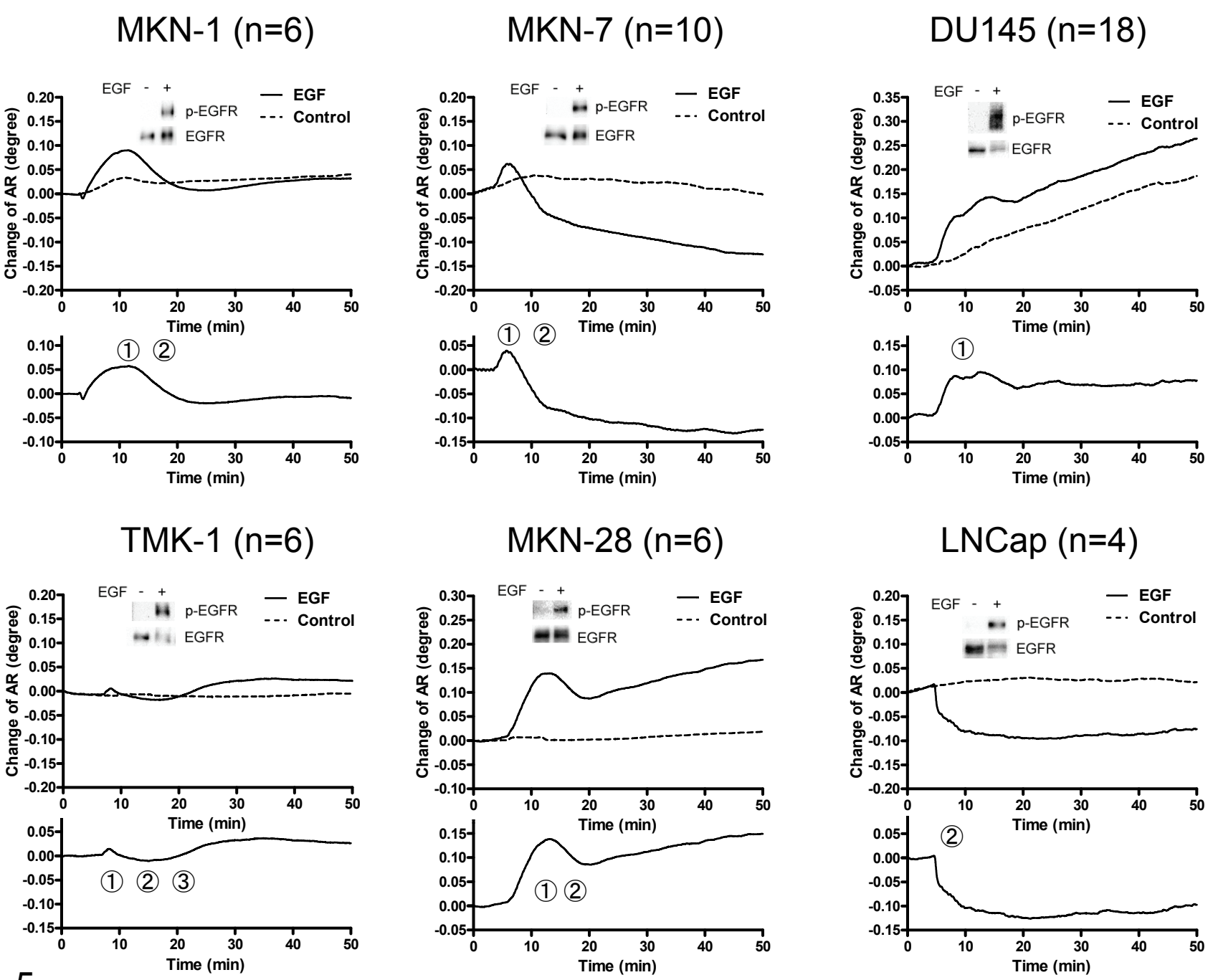
0.0
-0.1
-0.2
-0.3
-0.4
0
300
600
900
1200
1500
1800
Time (sec)
10 ng/ml
1 ng/ml

Fig. 4.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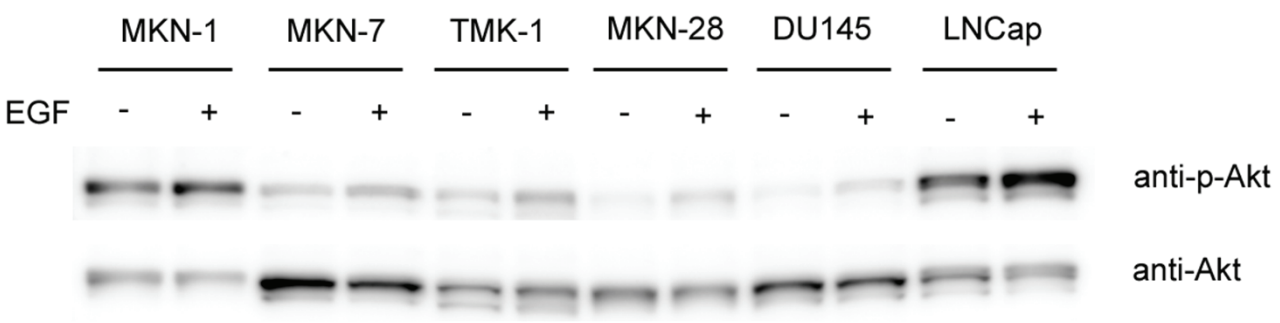
MKN-7 (n=10)
DU145 (n=18)
TMK-1 (n=6)
MKN-28 (n=6)
LNCap (n=4)
EGF - +
p-EGFR
EGFR
EGF
Control
Change of AR (degree)
Time (min)
① ② ③

Fig. 5.



Supplementary figure 1
The gastric cancer cell lines (MKN-1, MKN-7, TMK-1, and MKN-28) and the prostate cancer cell lines (DU145 and LNCap) were stimulated with 10 ng/ml of EGF for 15 min and then lysed. The whole cell lysates were immunoblotted for the detection of phosphorylated Akt or total Akt by the use of the specific antibodies against them. A representative data of three independent experiments is shown.