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Math1*, Retinoic Acid, and TNF α Synergistically Promote the Differentiation Of Mucous Cells in Mouse Middle Ear Epithelial Cells *In Vitro

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Abstract

Background—A key issue in otitis media (OM) is mucous cell metaplasia in the middle ear mucosa, a condition for hyperproduction of mucus in the middle ear mucosa and development of chronic OM. However, little is known about the driving force for the differentiation of mucous cells in OM.

Methods—Mouse middle ear epithelial cells (mMEEC) were used in this study to test whether *Math1*, a critical transcription factor for the development of mucous cells in the intestine, synergize with inflammatory cytokines (tumor necrotic factor alpha, TNF α) and other epithelial differentiation factors (retinoid acid, RA) to induce the differentiation of mMEEC into mucus-like cells in vitro. Simultaneously, *Math1* was transduced into the middle ear mucosa and see whether it induces mucous cell hyperplasia in vivo.

Results—*Math1* significantly increased the mucus cell numbers in the middle ear mucosa of mice. *Math1*, in the presence of TNF α and epithelial differentiation factor retinoic acid (RA), synergistically promoted the differentiation of mMEEC into mucus-like cells via upregulation of mucins and their chaperones:trefoil factors (TFFs) *in vitro*. RA treatment for 12 hours activated *Math1* although RA alone had very limited effects on mucus-like cell differentiation.

Conclusion—*Math1* plays a critical role in the pathogenesis of OM by induction of mucous cell differentiation, in the presence of TNF α and RA.

INTRODUCTION

Under normal conditions, few mucous cells exist in the middle ear mucosa of humans and rodents and they are mainly distributed in the orifice of Eustachian tube, promotary area,

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and inferior tympanium (so called “ciliated tract”). Under the pathological conditions, mucous cells are dramatically increased (1–7) with abundant production of mucins (2, 8) and mucin chaperones: trefoil factors (9). Upregulation of mucins and mucin chaperones is a biological basis for mucous cell metaplasia (MCM), a predisposition for chronic OM.

It is known that pro-inflammatory cytokines, such as TNF α , are involved in the development of MCM in animal models (10). However, little is known about the additional factors needed for the differentiation of mucous cells. Knockout of *Math1* (*Atoh1*, *atonal homolog 1*) in mice developed no mucous cell lineage in the intestine, suggesting that *Math1* is a candidate involved in the development of MCM in OM. Since the *Math1* knock out mouse model is embryonic lethal (11) and not available for MCM, we thus sought to investigate this issue by using *in vitro* “knock-in” techniques in mMEEC.

A classic pathway for MCM is middle ear infection. Inflammatory cells produce cytokines and cytokines, in turn, stimulate the differentiation of mucous cells (2, 10). A number of cytokines generated from inflammatory cells upregulate the expression of mucins and induce MCM. These cytokines include TNF α (10, 12–14), and interleukin-4 (IL-4),(15) IL-10,(16) IL-8,(17) IL-9,(18) IL-13 (19–21). They are either proinflammatory cytokines or lymphocyte (especially T helper 2 subset)-derived cytokines.

We hypothesized in this study that *Math1*, together with TNF α and RA, plays an important role in the differentiation of mucous cells in the middle ear. As expected, transfection of mMEEC with *Math1* regulated the expression of mucins and TFFs in the presence of TNF α and RA.

MATERIALS AND METHODS

Cell Culture

Mouse middle ear epithelial cells (mMEEC) were made in our laboratory as previously described (22). They were maintained in Ham’s F-12K culture media (American Type Culture Collection, ATCC, Manassas, VA) supplemented with 2 mM L-glutamine and 10 ng/ml epidermal growth factor (EGF), 5 μ L/ml insulin-transferrin-sodium selenite (ITS, 100 \times , Sigma-Aldrich, St. Lewis, MO) solution, 2.7 g/L glucose, 500 ng/ml hydrocortisone, 0.1 mM nonessential amino acids, 50 μ g/mL streptomycin, 50 units/mL penicillin and 4% fetal bovine serum, thereafter referred to as full growth medium (FGM, Sigma-Aldrich, St. Lewis, MO). FGM change was made every 3~4 days. During the experiment, EGF in the above media was omitted when various factors were added.

Regulation of *Math1* in mMEEC

mMEEC was cultured on 8-well chamber slides or in T25-flasks. Cells were starved in F-12K culture medium for 24 hours, then incubated with 20ng/ml EGF, 20ng/ml TNF α , 10^{-9} M RA, or TNF α +RA for 12 hours, and harvested for evaluation of *Math1* expression.

Transfection of *Math 1* in the middle ear mucosa of mice

Full-length *Math1* cDNA was cloned into a protein-expressing vector (C2 pEGFP, e.g., empty vector, Clontech, San Diego, CA), as previously described (23). The *Math1* cDNA

sequence in empty vector in a sense manner was confirmed by sequencing and referred to as sense-*Math1* cDNA (hereinafter referred to as *Math1*). To study the role of *Math1* in the differentiation of mucous cells, bilateral bullae of 5 mice were transfected with 10 μ L of *Math1* and empty vectors (ev), respectively, at 1.4 μ g/mL in Opti-MEM containing Lipofectin at 6 μ g/mL via the tympanic membrane approach. Transfected animals were sacrificed 7 days after *Math1* transfection for harvest of the bullae. The bullae were then fixed in 10% formalin and routinely processed for histological sections. Alcian blue-periodic acid Schiffs (AB-PAS) were employed for identification of mucous cells. Mucous cell numbers were quantitatively analyzed by the method as previously described (24).

Establishment of *Math 1* stably transfected mMEEC

In our pilot study, cDNA transfection efficiency of mMEEC was low and transient transfection was obviously insufficient for determination of the effect of *Math1* on cells. To overcome this problem, *Math1* and ev were stably transfected mMEEC. Briefly, cells were cultured in a 12-well plate to 70% confluence and transfected with *s-Math1* and ev at 1.4 μ g/mL for 16 hours in Opti-MEM (serum-free, Invitrogen, Grand Island, NY) containing 6 μ g/mL of Lipofectin, cultured in FGM, incubated with G418 (400 μ g/mL, Invitrogen, Grand Island, NY) for two weeks for selection of successfully transfected cells. This procedure was repeated once. Cells persistently resistant to G418 for 3 months were defined as stably transfected cells. To select highly positive cells for GFP, top 5% of GFP+ cells were sorted out by a cell sorter (FACSAria, BD Biosciences, Inc, Becton-Dickinson, Mountain View, CA) as described previously (25) after G418 selection twice. *Math1* mRNA and protein expression in these selected cell clones were routinely examined by RT-PCR, immunohistochemistry and/or fluorescence-activated cell sorting (FACS) for verification of *Math1* expression.

Induction of mucous cell differentiation in *Math1* stably transfected cells

Math1 and ev stably transfected cells were cultured for 24 hours in T-25 flasks or on 8-well chamber slides with a starting cell number at approximately 2.5×10^4 cells per well in starvation media (2% serum without any growth factors). Cells were incubated with 20ng/mL of TNF α , 10^{-9} of M RA, or TNF α +RA, respectively, for two weeks in FGM (media and factors were supplied every two days) and then starvation media for 2 days. Cells were then harvested for evaluation of mucous cell differentiation by AB-PAS, immunohistochemistry, and FACS.

Inhibition of mucous cell differentiation with specific inhibitors

To study which signaling pathways are involved in mucous cell differentiation, *Math1* and ev stably transfected cells were pre-incubated pathway inhibitors (25 μ M PD98059 for the Erk pathway, 20 μ M LY294002 for the Akt pathway, and 10 μ M AG1478 for the EGFR pathway from Calbiochem-Novabiochem, San Diego, CA, 15 μ M SB203580 for the p38 MAPK pathway and 20 μ M SP600125 for the JNK pathway from Sigma-Aldrich, St. Lewis, MO) for two hours and incubated with TNF α , RA, and TNF α +RA for 14 days in the presence of the above inhibitors, followed by the above starvation media for 2 days without

TNF α , RA, and TNF α +RA but with inhibitors. Cells were harvested for evaluation of mucous cell differentiation by AB-PAS, immunohistochemistry and FACS.

AB-PAS stain

Cells on chamber slides were fixed with 100% ethanol for 6 minutes in room temperature and incubated with 1% alcian blue (AB) for 30 minutes, 0.5% PAS reagent for 10 minutes, then sulfurous acid for two minutes. Slides were washed in tap water and examined under a light microscope for identification of positive mucous granules. Cells with blue and purple colors were defined as mucous cells. Mucous cell numbers from 4~6 areas on each chamber were counted. Quantitative data are presented as mean \pm S.E. Total cell numbers were determined by DAPI stain simultaneously and the percentage of AB-PAS positive cells against total cells was calculated. Final mucous cells are presented as % of total cell numbers

Immunohistochemistry

Cells on Lab-Tec chamber slides (Nalge Nunc International, IL) were fixed in 100% ethanol, and incubated with mucous cell marker antibodies: anti-TFF3(1:50, Calbiochem, San Diego, CA) or anti-Math1 (1:100, abcam, Cambridge, MA) at 4°C overnight, washed with PBS, incubated with fluorescein isothiocyanate (FITC)- or tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibodies (Zymed, Santa Cruz, CA) for 60 minutes at room temperature, washed with PBS, and examined under a fluorescence or a confocal microscope. Non-specific IgG was used for immunohistochemistry negative control.

Fluorescence-Activated Cell Sorting (FACS)

Cells were incubated with 0.3% saponin in PBS for 5 minutes, incubated with anti-TFF3 (1:50) or anti-Math1 (1:50) for 30 minutes on ice, washed with 0.3% saponin in PBS twice, incubated with TRITC-conjugated secondary antibody for 30 minutes on ice, washed with 0.3% saponin in PBS twice, and analyzed on a BD FACSCalibur flow cytometer. Cells incubated with non-specific IgG (zymed products), followed by TRITC-conjugated secondary antibodies, served as antibody controls. Data was analyzed with CellQuest Pro (BD Sciences) and FlowJO (7.1 version, Tree Star Inc) for presentation. Experiments were run in duplicate or triplicate. Results are presented as % positive cells over 3,000 cells per sample after subtraction with non-specific IgG background.

RT-PCR

Total RNA was isolated from the above harvested cells using RNA Miniprep Kit (Stratagene). Residual genomic DNA in total RNA samples was digested with DNases according to the manufacturer's instruction. Specific primers for *Math1*, TFF3, GAPDH were as follows: *Math1* primers: 5'-agatctacatcaacgctctgtc-3'/5'-actggcctcatcagagtcactg-3' (58.5°C, 452bp, 30cycles); TFF3 primers: 5'-cagattacgttggcctgtctcc-3'/5'-atgcttctacccttgaccac-3' (60°C, 254bp, 30cycles); and GAPDH primers: 5'-aacgggaagcccatcacc-3'/5'-cagccttggcagcaccag-3' (61°C, 441bp, 20cycles). The specificity of these primers was by BLAST search using the National Center of Biologic Information

web site (<http://www.ncbi.nlm.nih.gov>). RT-PCR was performed as described previously (26, 27). PCR products were analyzed on a 2% agarose gel for evaluation of band sizes and routinely purified for sequence verification. RNA samples omitting reverse-transcription enzyme served as controls.

Microarrays

Affymetrix microarrays were performed using the above total RNA as previously described (28). Briefly, cDNA was prepared from 20 µg total RNA using a T7-dT₂₄ primer. cRNA was synthesized from cDNA and biotinylated using the BioArray High Yield RNA Transcript Labeling Kit (Enzo Diagnostics, Farmingdale, NY) and hybridized with the Rat U34 arrays (Affymetrix, Santa Clara, CA). The microarray data were analyzed as previously described (28).

RESULTS

Math1 transfection induces mucous cell metaplasia in the middle ear mucosa of mice

To study the importance of the *Math1* gene in differentiation of mucous cells, transfection of *Math1* in the middle ear was performed in the 10 bullae of 5 mice. It was found that mucous cell numbers were increased in *Math1* transfected middle ear mucosa (Fig 1A) compared with empty vector transfected middle ear mucosa (Fig. 1B). Statistically, AB-PAS positive cells were significantly higher in the bullae transfected with *Math1* than those transfected with empty vector (Fig. 1C).

Math1 transfection alone in vitro dose not induce mucous cell differentiation

To investigate the role of *Math1*, cultured mMEEC were stably transfected with *Math1* and empty vector, respectively. After incubation with G418 for more than 3 months, survived cells were submitted for selecting GFP highly positive cells. *Math1* was weakly expressed in empty vector-transfected cells. *Math1* transfection successfully increased the expression of *Math1* by RT-PCR, FACS, and immunohistochemistry (Supplemental Figure 1, online), suggestive of efficient transfection of *Math1* in cultured mMEEC. To study whether *Math1* is involved in the development of mucous cells, *Math1* and empty vector stably transfected cells were incubated with various factors for 14 days and then without these factors for 2 days before harvesting for evaluations. Microarrays data confirmed that *Math1* alone increased the mRNA of mucins (including Muc1, Muc2, Muc4 and Muc5ac but not Muc3 and Muc10), and mucin shaperones (TFF1 and TFF2 but not TFF3) compared with empty vector (Fig. 2). However, *Math1* transfection alone did not significantly increase the AB-PAS positive cell numbers (Fig. 3A), nor the expression of Muc2 protein in the cells as judged by immunohistochemistry (Fig. 3B). *Math1* increased the % of Muc2 and TFF3 positive cells when used together with TNF α and RA in comparison with empty vector as judged by FACS (Fig. 3C).

RA+TNF α +Math1 synergistically induces formation of mucous granules in cultured mMEEC

Under inflammatory conditions, various cytokines and factors exist in the middle ear. To study whether they synergize in triggering the differentiation of mucous cells, mMEEC cell

cultures were incubated with and without inflammatory cytokines and factors for 14 days, then without any factors for 2 days to allow them to differentiate. It was found that TNF α +RA were able to synergistically increase the number of AB-PAS positive cells whereas RA and TNF α alone were unable to do so (Supplemental Figure 2, online). To study whether *Math1* is involved in the differentiation of mucous cells under inflammatory conditions, *Math1* and empty vector stably transfected cells were incubated with TNF α +RA for 14 days and then without TNF α +RA for 2 days. It was found that TNF α +RA+*Math1* had significantly higher AB-PAS positive cell numbers compared with TNF α +RA+empty vector (Fig. 3A). Immunohistochemistry verified that TNF α +RA+*Math1* obviously increased the expression of both Muc2 and TFF3 in cells compared with controls (empty vector, *Math1*, RA+*Math1*, and TNF α +*Math1*, Fig. 3B). It has been noted that translocation of Math1 protein to the nucleus was observed in the presence of RA and/or TNF α (namely, R*Math1* or T*Math1*). While *Math1* transfection alone did not have sufficient Math1 protein in the nucleus (*Math1*), suggesting that TNF α and/or RA activate Math1 in the nuclei. This explains why TNF α +RA+*Math1* cocktail is essential for induction of mucous cell differentiation. FACS showed that *Math1* transfectants had an increase of the % of both Muc2 and TFF3 positive cells compared with empty vector transfectants in the presence of TNF α +RA (Fig. 3C), namely, TNF α +RA+*Math1* increased the % of both Muc2 and TFF3 positive cells. It is noted that Muc2 positive cells were also TFF3 positive cells (Fig. 3C).

RA activates Math1 in mMEEC

To study whether RA activates Math1, cells were incubated with various factors (with and without RA, TNF α , and TNF α +RA for 2~48 hours) and harvested for RT-PCR, immunohistochemistry, and FACS. It was demonstrated that RA treatment for 12~24 hours increased the expression of Math1 mRNA transcripts by RT-PCR (Fig. 4A). PCR negative controls were all negative. FACS demonstrated that RA, alone or in combination with TNF α , increased the % Math1-positive cells (Fig. 4B). Immunohistochemistry verified that RA increased the expression of the Math1 protein in a time-dependent manner and translocated into the nuclei (Fig. 4C).

Multiple signaling pathways are involved in mucous cell differentiation by TNF α +RA +*Math1*

It remains unclear how RA, TNF α , *Math1* alone and in combination affect differentiation of mucous cells. To probe the likely signaling pathways for mucous cell differentiation, we evaluated the effects of various chemical inhibitors on mucous granule formation. All the inhibitors except for JAK1 significantly inhibited TNF α +RA+*Math1* induced mucous granulation in cultured mMEEC (Fig. 5A). Immunohistochemistry verified that in comparison with DMSO, PD98059, SB203580, LY294002, AG1478, and SP600125 reduced the expression of mucous cell marker TFF3 in *Math1* stably transfected and TNF α +RA -treated cells (Fig. 5B). Taken together, the data showed that multiple pathways are involved in the differentiation of mucous cells induced by TNF α +RA+*Math1*.

DISCUSSION

In this study we demonstrated for the first time that mMEEC have the potential to differentiate into mucous-like cells under certain circumstances. The inductive factors are triad master regulators for mucous cell metaplasia: *Math1*, TNF α , and RA. There is an expression baseline of *Math1* in mMEEC. RA or retinoids are also available in the middle ear mucosa. Under normal conditions, these mMEEC become epithelial cells, instead of mucous cells. But under inflammatory conditions, TNF α and other cytokines are induced and released. This results in the synergy of the above three important factors: *Math1*, TNF α , and RA on the middle ear epithelial cells. *Math1* alone has some effects on the expression of mucous cell markers such as mucins and TFFs at the mRNA level (Fig. 2) but limited at the protein level (Fig. 3). *Math1*, TNF α , and RA synergistically regulate mucins and mucin chaperones (TFFs) at the both mRNA and protein levels. This indicates that mucous cell metaplasia requires converged signaling pathways from multiple factors (Fig. 6). Mucous granules are formed when *Math1*, RA, and TNF α reach to a certain level, so called threshold for MCM. These mucous granules are AB-PAS positive and cells containing abundant mucous granules and they are mucous cells by nature. Rather, these cells *in vitro* are not mature mucous cells but are *en route* to become mucous cells.

How TNF α , RA and *Math1* make this happen? First, each of these factors plays a role in the regulation of mucins: hallmark proteins for mucous cells. These upregulated mucins are multiple: soluble mucins such as Muc2, Muc5ac, Muc5b and membrane-bound mucins such as Muc1 and Muc4. *Math1* has an potential effects on the expression of Muc1, Muc2, Muc4 and Muc5ac, but not Muc3, Muc10, based upon the microarray data (Fig 2) and, in fact, *Math1* transfection increases the expression of Muc2 protein in the presence of RA +TNF α (Fig 3C). Consistent with this, Leow and coworkers reported that *Math1* is related to MUC2 mucin upregulation (29). Sekine and coworkers reported that overexpression of *Math1* in the gastric cancer cells enhanced MUC5AC mRNA and knockdown of *Math1* by RNA interference decreased MUC5AC gene (30). In our previous studies, TNF α stimulated the expression of mucins (Muc2) in the rat middle ear mucosa and promotes the differentiation of mucous cells (12). In line with this, TNF α *in vitro* regulates the expression of Muc2 (Fig 3B), and slightly stimulates the differentiation of mucous cells *in vitro*. In terms of RA, it has been reported that the expression of MUC2, MUC5AC and MUC5B are dependant on the presence of RA because these mucin mRNA transcripts were not detected in the RA-deficient cultures (31). Our current study demonstrated that RA treatment induces the expression of Muc2 as judged by immunohistochemistry(Fig 3B). Second, in the presence of *Math1*, TNF α and/or RA regulate the expression of mucin (Muc2) and mucin shaperones TFF3 (Fig. 3B). TFFs are essential for packing mucins into mucus granules (9) which stain positive for AB-PAS. Neither mucins nor TFFs alone make any mucous granules, so the upregulation of both mucins and TFFs at the same time is a prerequisite for the formation of mucous granules and differentiation of cells into mucous-like cells. *Math1* transfection had increased the expression of *Math1* in the cytosol. However, these upregulated protiens were not translocated to the nuclei without presence of TNF α and/or RA (Fig 3B). This may explain why *Math1* transfection alone has a limited or no effect on the expression of TFF3 (Fig. 2). *Math1* is thought to synergize the expression of TFF3

because *Math1* transfection strengthened the effects of TNF α +RA on TFF3 expression (Fig 3C). The pathway to TFF3 is still poorly understood. It has been reported that the PI3-K/Akt pathway links to TFF3 expression in TH29 cells under confluence (32). Consistent with this, an Akt specific inhibitor, LY294002, inhibited the expression of TFF3 by TNF α +RA(Fig. 5B). TFFs are known to stimulate their own release as well as other family members and this auto- or cross-induction of TFFs requires indirect activation of the EGFR (33). This may be one of reasons why EGFR tyrosin kinase inhibitor, AG1478, inhibited the expression of TFF3 by TNF α +RA(Fig 5B). Our microarray data shown the remarkable upregulation of AKT(AF124142) and EGFR was observed in TNF α +RA+*Math1* treated cells (Fig. 2). These signaling pathways induced by the combination of factors may explain the synergistic effects of TNF α + RA+*Math1* on TFF3 expression or mucous cell differentiation.

RA has been shown to regulate epithelial cell differentiation related to mucin gene regulation in rabbit tracheal-epithelial cells *in vitro* (34) and restore to a mucous cell phenotype from squamous cells when cells are derived of retinoids (35). In this study, we demonstrate that RA mainly regulates the expression and activation of *Math1*, which further potentiate the expression of both mucins and mucin chaperones. The upregulation of mucins and mucin chaperones to a certain level may trigger the differentiation of epithelial cells to mucous cells. Although either TNF α or RA alone is capable of upregulating the expression of mucins or TFF3 *in vivo*, it is not sufficient for differentiation of mucous cells in a cell culture system. *Math1*, TNF α , and RA synergistically promote mucous cell differentiation *in vitro*, suggesting that they are therapeutic targets for mucous cell metaplasia in OM or asthma in the airway.

It is poorly understood how many signaling pathways are involved in the differentiation of mucous cells. The data collected from the current study indicate that the complex effects of *Math1*+TNF α +RA on mucous cell differentiation are blocked by several specific inhibitors such as SB203580, LY294002, AG1478, and SP60012. It suggests that TNF α +RA+*Math1* synergized mucous cell differentiation is a result of the interactions between different pathways. Cross-talks between cytokines and factors are essential for mucous cell differentiation. Further studies are warranted to clarify the specific signaling pathways for individual cytokines in order to design specific therapeutic interventions relevant to inflammatory cytokines.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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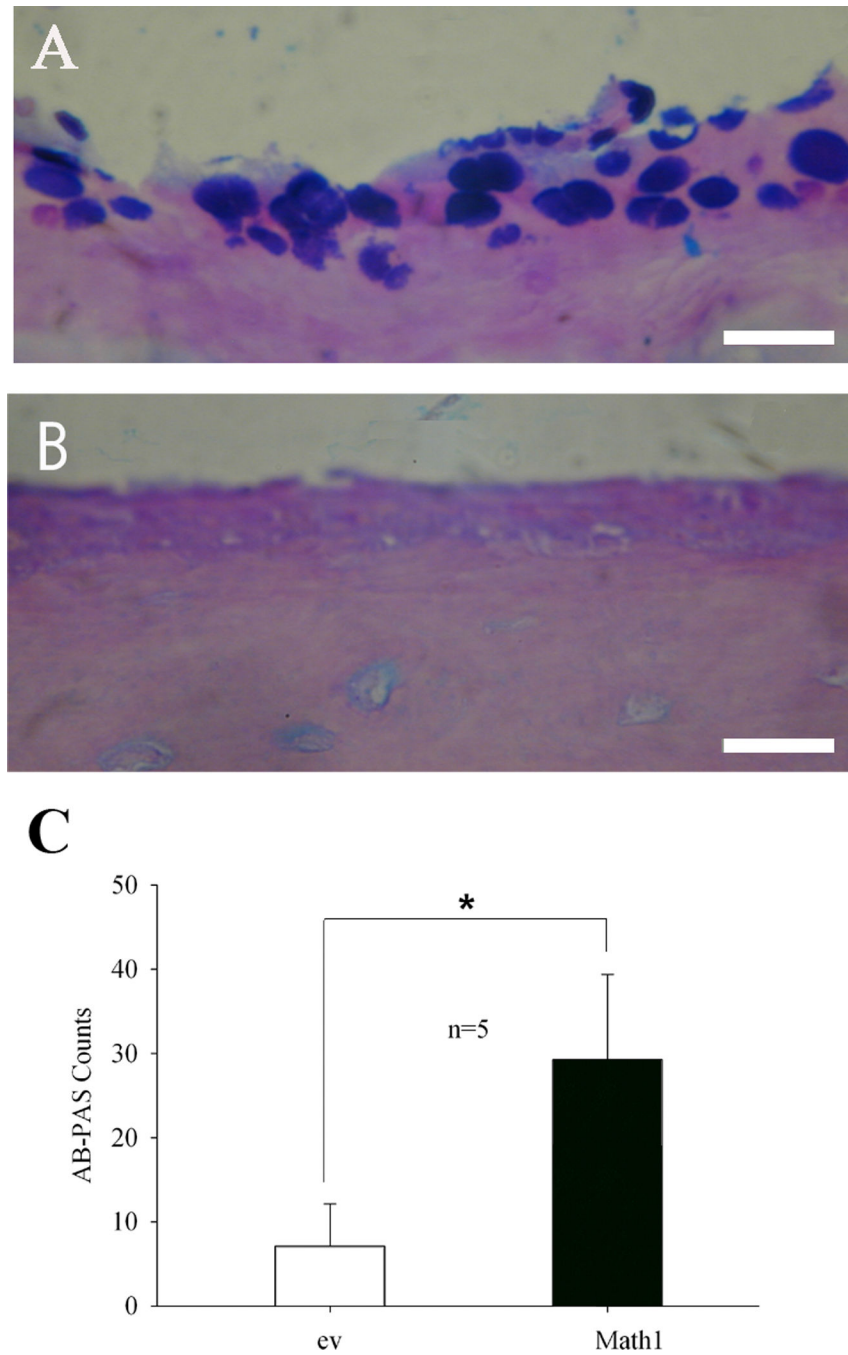


Fig. 1. *Math1* transfection increases the mucous cell numbers in the middle ear mucosa of mice. *Math1* transfection had more AB-PAS positive cells in the mouse middle ear mucosa (A) than empty vector (B) transfection. Statistical analysis showed that *Math1* significantly increased the AB-PAS cell counts in the middle ear mucosa of 5 mice (C). * $p < 0.01$ ($n = 5$); Bar = 10 μm .

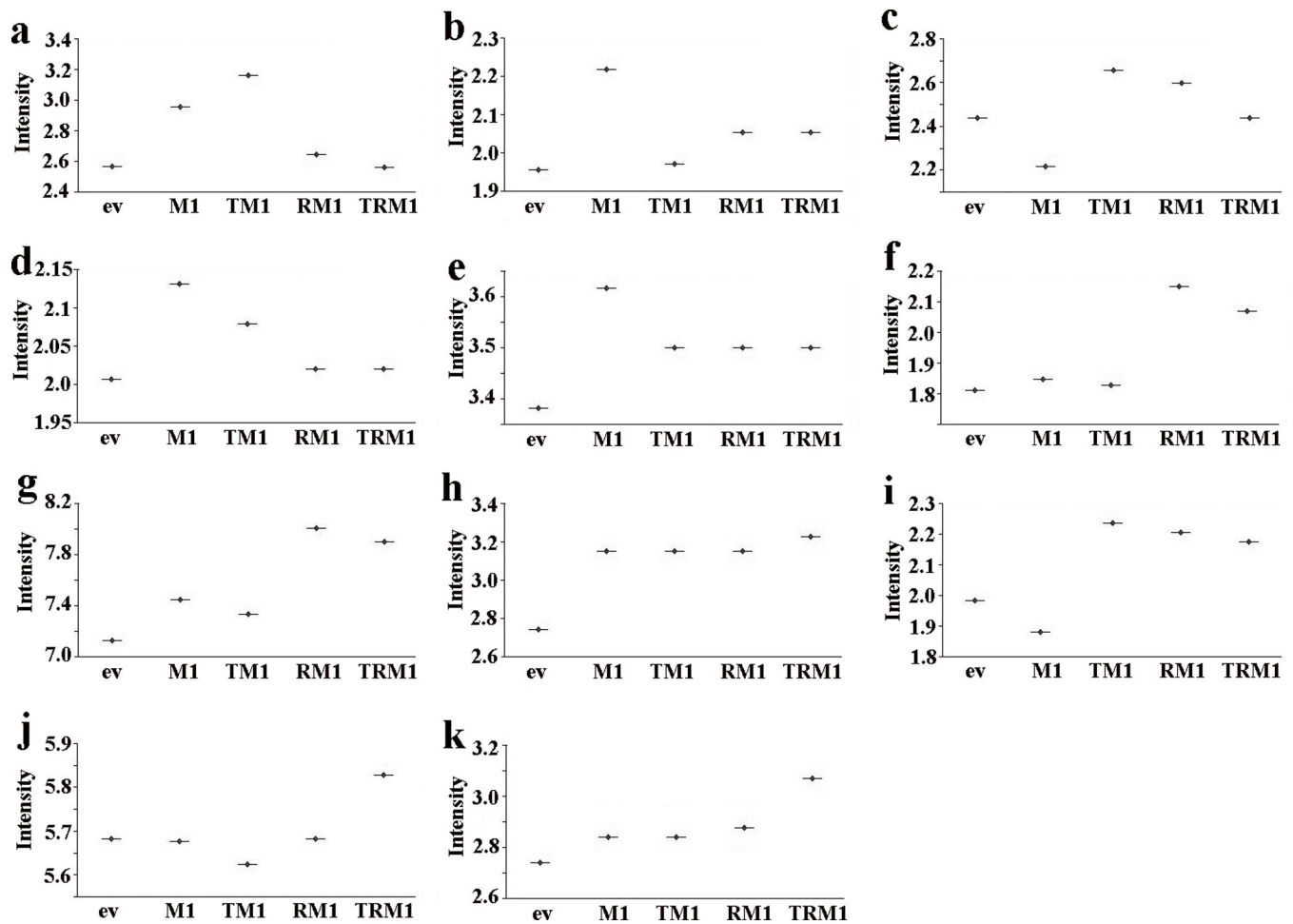


Fig. 2.

Math1 regulates the expression of mucous cell markers at the mRNA level in cultured mMEEC. Microarray data confirmed that the upregulation of mucins: Muc1(a), Muc2 (b), Muc4 (d), Muc5ac (e), but not Muc3 (c) and Muc10 (f) and mucin chaperones: TFF1(g) and TFF2 (h) but not TFF3 (i) at the mRNA level. TNF α +RA+*Math1* (TRM1) obviously increased the expression of TFF1 (g), TFF2 (h), TFF3 (i), EGFR (j), and AKT (k) at the mRNA level. ev, empty vector; TM1, TNF α +*Math1*; RM1, RA+*Math1*; TRM1, TNF α +RA+*Math1*.

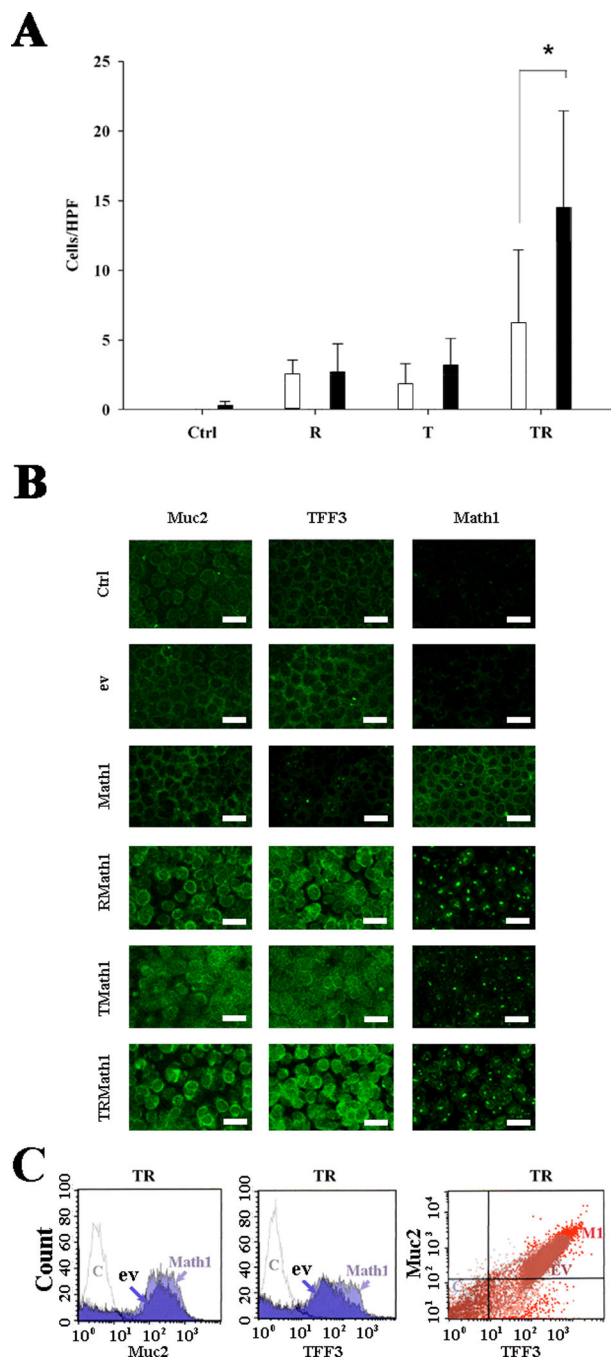


Fig. 3. TNF α +RA+*Math1* synergistically increased mucous granules (AB-PAS positive cells) in cultured cells. Cells were treated with various factors for 14 days and then without these factors for 2 days. RA and TNF α alone induced few AB-PAS positive cells and *Math1* transfection alone did not significantly increase the AB-PAS positive cells compared with empty vector (A, AB-PAS staining). However TNF α +RA+*Math1* significantly increase the % of AB-PAS positive cells compared with TNF α and RA (A, *p<0.05, n=6). empty bar, ev; solid bar, *Math1*; T, TNF α ; R, RA; TR, TNF α +RA. Immunohistochemistry showed that

TNF α +RA+*Math1* cocktail obviously increased the expression of both Muc2 and TFF3 (B). $\bar{=}$ 10 μ m. FACS verified that *Math1* transfection increased the % positive cells for Muc2 and TFF3 compared with empty vector in the presence of RA and TNF α , and cells positive for Muc2 were also positive for TFF3 (C). HPF, high power field; Ctrl, control (untransfected cells); ev, empty vector; T*Math1*, TNF α +*Math1*; R*Math1*, RA+*Math1*; TR*Math1*, TNF α +RA+*Math1*.

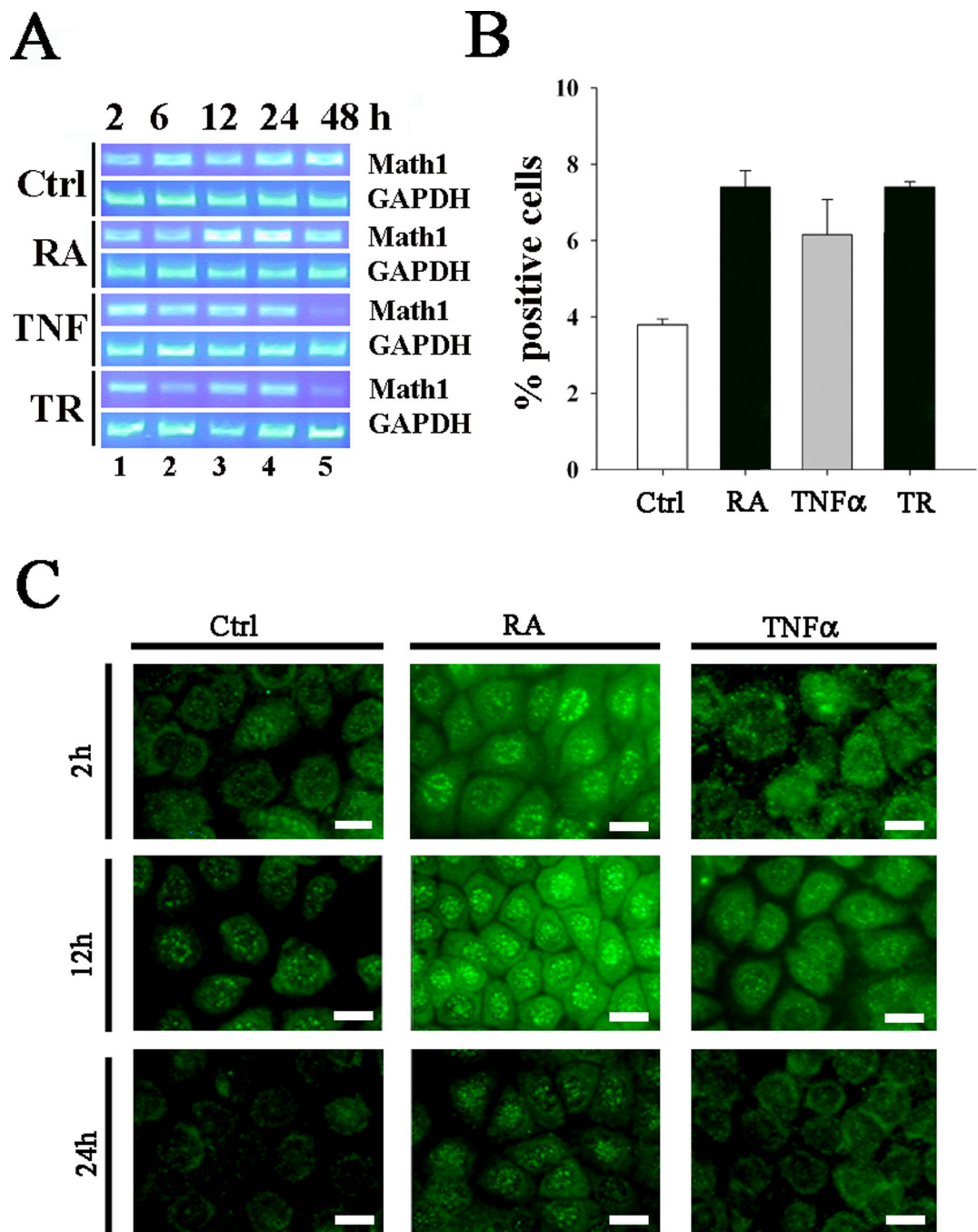


Fig. 4. RA and RA+TNF α strengthen the expression of *Math1* in cultured mMEEC. Incubation of cells with RA for 12~24 hours increased the expression of the *Math1* mRNA transcripts by RT-PCR (A). FACS verified that RA, TNF α , and RA+TNF α for 12 hours increased *Math1*-positive cell numbers (B, n=2). Immunohistochemistry further confirmed that an increase of the *Math1*-positive cells and translocation into the nuclei after treatment with RA and TNF α in a time-dependent manner (within 2~12 hours, C). It is noted that both RA and TNF α had

no effects on the Math1 translocation to the nuclei at 24 hour. EGF=20ng/mL, TNF α =20ng/mL, RA=1 μ M. Ctrl, control; bar=5 μ m.

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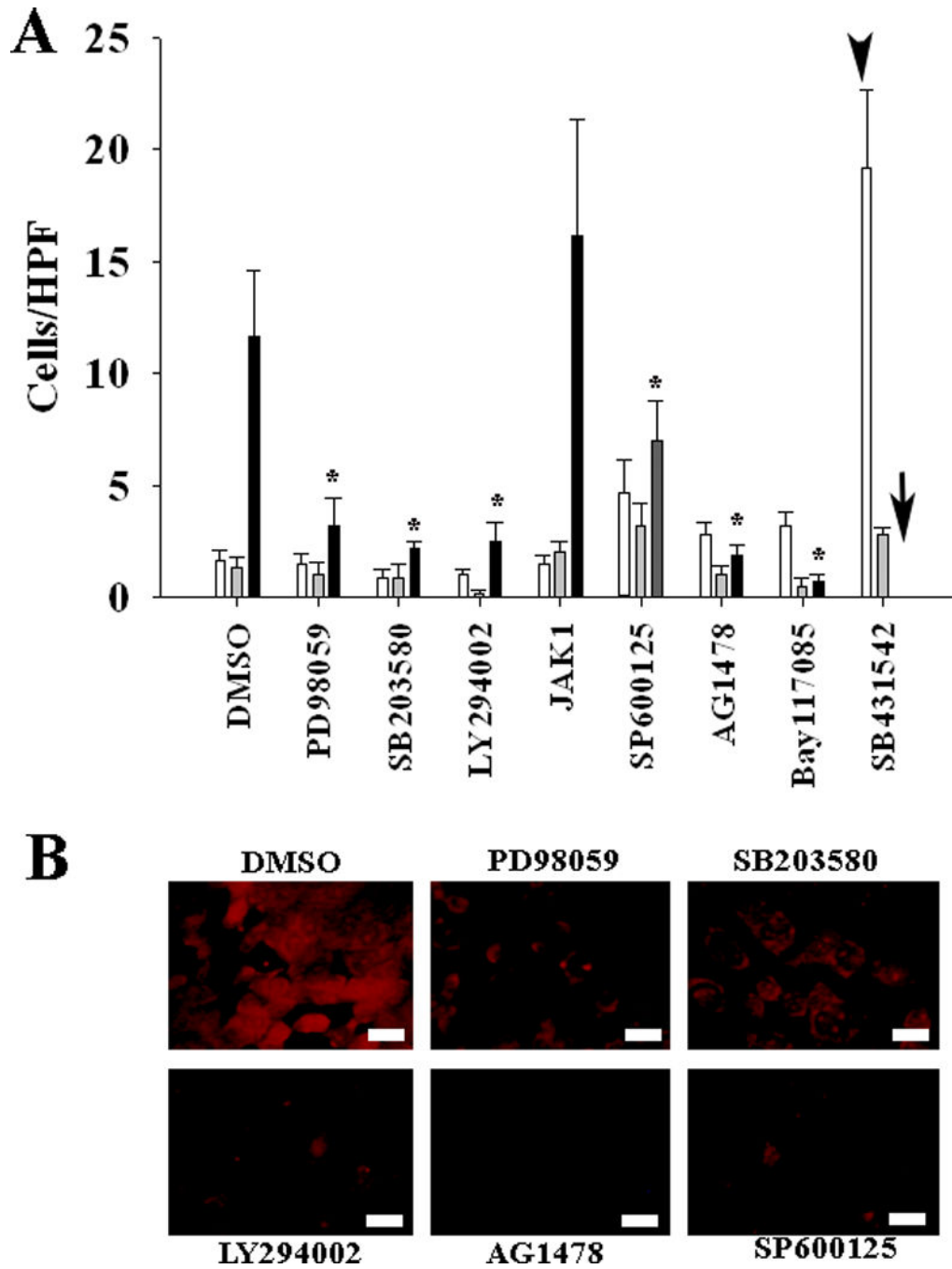


Fig. 5. Multiple signaling pathways are involved in mucous cell differentiation by $TNF\alpha$ +RA+*Math1*. All the inhibitors used in this study, except JAK1, significantly inhibited the mucous granule formation induced by $TNF\alpha$ +RA+*Math1* (A, white bar, TMath1; grey bar, RMmath1; black bar, TRMath1). JAK1 did not affect the mucous cell differentiation triggered by $TNF\alpha$ +RA+*Math1* whereas SB431542 did not affect the mucous cell differentiation triggered by $TNF\alpha$ +*Math1* (arrow head) but toxic to $TNF\alpha$ +RA+*Math1* treated cells (black arrow). Immunohistochemistry demonstrated that PD98059, SB203580, LY294002,

AG1478, and SP600125 inhibited the expression of TFF3 in TNF α +RA+*Math1* treated cells (B). bar=5 μ m. *p<0.05 (n=6)

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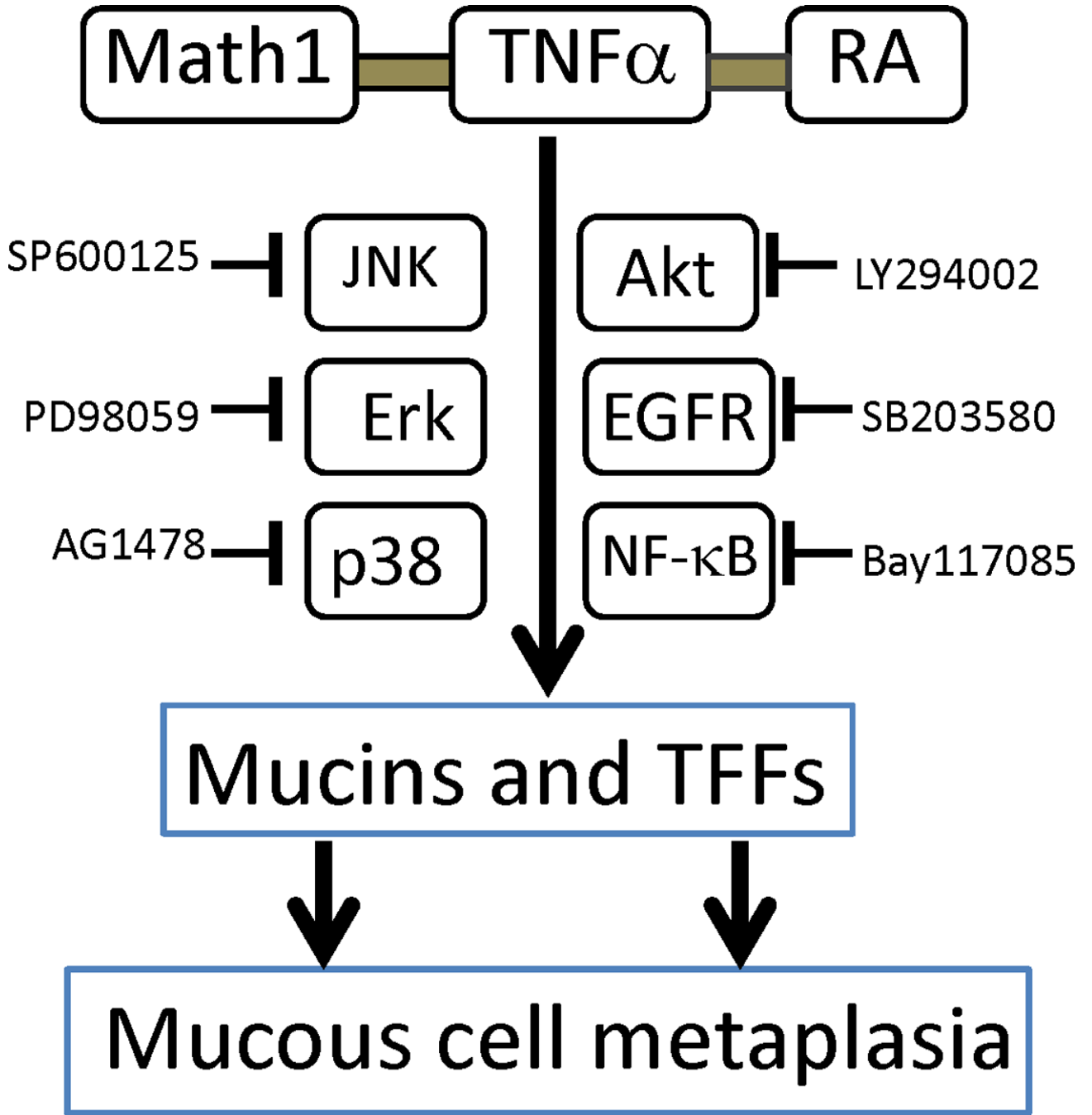


Fig. 6. A flow chart summary for the signaling pathways involved in mucous cell metaplasia by TNFα+RA+Math1 in mMEEC. As judged by AB-PAS and immunohistochemistry data (see Fig. 5), the Erk, p38MAPK, Akt, EGFR, JNK and NF-κB signaling pathways are involved in TNFα+RA+Math1 induced mucous-like cells (AB-PAS positive). In other words, Math1, TNFα and RA may act through the above signaling pathways synergistically to increase the proliferation and differentiation of mucous cells in cultured mMEEC and

blockage of the above pathways may lead to therapeutic interventions of mucous cell metaplasia in the middle ear milieu.

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