

Abrogation of Tumor Necrosis Factor- α converting enzyme inhibits embryonic lung morphogenesis in culture

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ABSTRACT TNF- α converting enzyme (TACE)-mediated cell surface protein ectodomain cleavage constitutes an important cellular regulatory mechanism during mammalian lung development. Herein, we have found that TAPI, a synthetic inhibitor of TACE, inhibits embryonic mouse lung branching morphogenesis in culture. To further investigate the biological significance of TACE as a shedding enzyme during early lung organogenesis, we have devised an antisense oligonucleotide to specifically block endogenous TACE gene expression at both transcriptional and translational levels in embryonic mouse lung explant culture. Addition of TACE antisense oligonucleotide resulted in a concentration-dependent reduction in lung branching morphogenesis in culture, whereas both scrambled and sense control oligonucleotides showed no adverse effects on lung growth. Furthermore, both aquaporin-5 (Aqp5) and surfactant protein-C (SP-C) mRNA expression and protein immunoreactivity were significantly inhibited in cultured mouse lungs treated with TACE antisense oligonucleotide, indicating defective epithelial cell differentiation in embryonic lungs with decreased TACE expression. TACE is known to be involved in the proteolytic release of TGF- α , an EGF family stimuli critical for lung growth and maturation. We therefore tested the possibility that a lack of diffusible TGF- α , due to TACE deficiency, contributes to the inhibitory lung morphogenesis in the presence of TACE antisense oligonucleotide in lung culture. Soluble TGF- α , when included in the lung culture, rescued the TACE antisense oligonucleotide-treated lungs from inhibition of both lung branching morphogenesis and lung epithelial cell differentiation, suggesting an impaired release of circulating regulators necessary for lung development in the absence of TACE gene expression. Our findings provide evidence that TACE-mediated membrane protein shedding is indispensable for normal lung branching morphogenesis and cytodifferentiation, probably through regulating the availability of positive cytokines/growth factors essential for lung organogenesis such as TGF- α .

KEY WORDS: TACE, TGF- α , lung branching morphogenesis, TAPI, antisense oligonucleotide, lung explant culture

Introduction

Metalloprotease-disintegrins are a family of transmembrane glycoproteins that play critical roles in cell-cell interactions and in the processing of the protein ectodomain cleavage. They are characterized by a conserved domain structure, consisting of an N-terminal signal sequence followed by a prodomain, metalloproteinase and disintegrin domains, a cysteine-rich region, a transmembrane domain and a cytoplasmic tail (Müllberg *et al.*, 2000). Thus, these enzymes are grouped into ADAM family (a disintegrin and metalloproteinases) (Black and White, 1998).

ADAMs are involved in proteolytic processing of EGF-like molecules (Schlöndorff and Blobel, 1999; Leserer *et al.*, 2000).

These membrane-associated protein precursors are subjected to limited proteolysis giving rise to soluble forms containing the entire extracellular domains of the proteins, a process termed shedding. The first and best-characterized "sheddase" is TACE (tumor necrosis factor alpha converting enzyme, also referred as ADAM17) (Black *et al.*, 1997; Moss *et al.*, 1997). Besides TNF- α , TACE enzymatically cleaves several other membrane proteins, such as TGF- α , L-selectin, p75 TNFR, and HER4 (Peschon *et al.*, 1998; Rio *et al.*, 2000). Since many cytokines and growth factors

Abbreviations used in this paper: Aqp5, aquaporin-5; SP-C, surfactant protein C; TACE, TNF- α converting enzyme; TGF, transforming growth factor; TNF, tumor necrosis factor.

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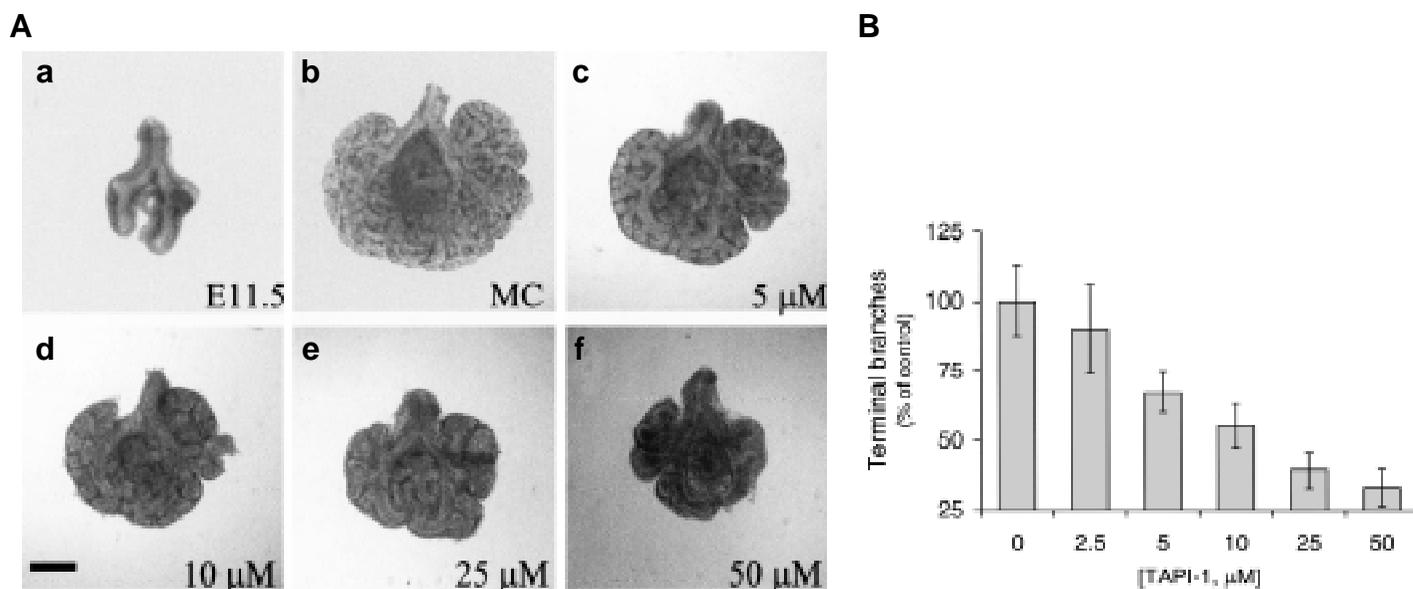


Fig. 1. TAPI-1 inhibits embryonic mouse lung branching morphogenesis in culture. **(A)** (a) An E11.5 mouse lung explant underwent extensive morphogenesis to develop into (b) a characteristic branching pattern after 4 days in serumless culture. Inhibition of branching morphogenesis was observed in lungs in the presence of different doses (5, 10, 25, and 50 μM for (c,d,e,f), respectively) of TAPI-1. Bar, 100 μm. **(B)** Addition of TAPI-1 inhibited lung branching morphogenesis in a concentration-dependent manner, as determined by the number of peripheral branches in cultured embryonic mouse lungs.

are initially synthesized as transmembrane precursor proteins which have to be cleaved in order to reach into the circulation, it seems that TACE-mediated protein secretion may regulate the signaling of the cognate growth factors and cytokines (Leserer *et al.*, 2000).

The primitive lung epithelium undergoes cell proliferation, branching morphogenesis, and alveolar saccular formation as well as simultaneous cell lineage differentiation to form an organ capable of conducting respiratory gases to a large, diffusible interface with the circulation (Hilfer, 1996). Lung growth and development is currently known to take place within a complex milieu of peptide growth factors and cytokines, which affect many parameters of cell behaviors including proliferation, differentiation, migration, interaction, survival, and extracellular matrix deposition (Warburton *et al.*, 2000). It is therefore likely that soluble factors including growth factors and cytokines instruct lung organogenesis by coordinated temporo-spatial signal transduction. However, despite the fact that TACE-mediated ectodomain shedding represents an important and efficient strategy to regulate the availability of intercellular proteins in the developing mammalian tissues, the biological significance of TACE gene expression during early lung growth and development is completely not understood.

In the current report, the biological role of TACE during lung development was elucidated using an *ex vivo* embryonic lung explant culture undergoing branching morphogenesis, in which TACE gene is expressed during the culture period. While TACE-mediated protein ectodomain shedding triggers signal transduction of cognate pathways, we postulated that TACE gene expression could regulate lung morphogenesis through availability of soluble cytokines/growth factors. To test this hypothesis, we utilized an antisense oligodeoxynucleotide to abrogate endogenous TACE gene expression as a "loss-of-function" approach during embryonic lung morphogenesis in culture. We discovered that both lung branching and cytodifferentiation were inhibited

when endogenous TACE gene expression was abolished using antisense oligonucleotide in embryonic lungs in culture. Mice lacking TACE showed a similar phenotype to animals engineered to lack TGF- α or EGFR, and their fibroblasts were defective in shedding of not only TNF- α but also TGF- α , suggesting the involvement of TACE in membrane cleavage of EGF-like ligand precursors (Miettinen *et al.*, 1995; Peschon *et al.*, 1998; Luetke *et al.*, 1999). We have further demonstrated that addition of TGF- α , as a soluble stimulatory factor, complemented TACE antisense oligonucleotide-treated lungs from both inhibited branching and defective epithelial differentiation in embryonic lung explant culture. Therefore, our results defined a novel concept that TACE-mediated membrane ectodomain shedding is responsible for normal lung growth and development.

Results

TAPI-1 inhibits lung branching morphogenesis in a dose-dependent manner in culture

Since the metalloproteinase inhibitor TAPI (TNF- α protease inhibitor) is known to prevent spontaneous membrane ectodomain shedding including that of TNF- α (Mohler *et al.*, 1994), we sought to investigate the possibility that TAPI regulates lung morphogenesis. E11.5 embryonic mouse lung explants were cultured for 4 days in serumless, chemically-defined BGJb medium in the presence of various doses of TAPI-1 compound. As shown in Fig. 1, TAPI-1, when added exogenously to culture medium, inhibited lung branching morphogenesis in a concentration-dependent manner (2.5–50 μM). At a concentration of 50 μM TAPI-1, lung branching was almost arrested, as quantification of peripheral branches revealed that TAPI-1 at above dose decreased embryonic lung branching by a significant 68% ($P < 0.05$). Therefore, inactivation of metalloproteinases including TACE, using TAPI, resulted in the inhibited lung branching morphogenesis in culture.

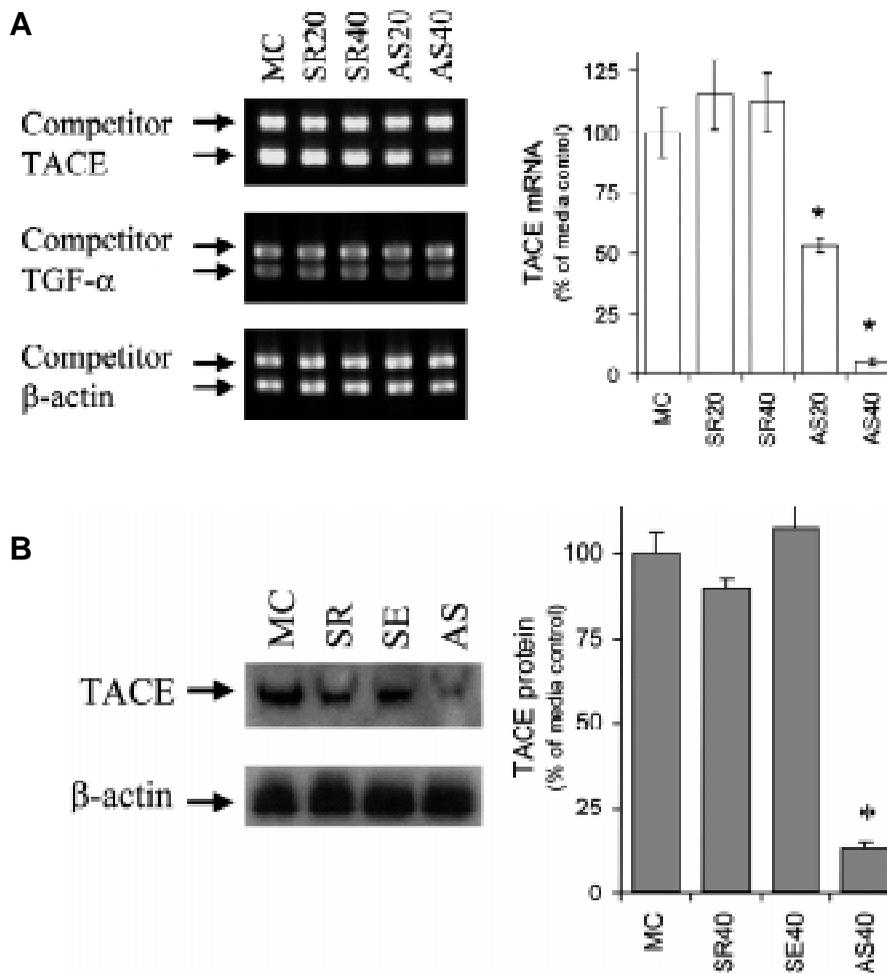


Fig. 2. TACE antisense oligonucleotide inhibits TACE gene expression at both mRNA and protein levels in cultured embryonic mouse lungs. (A) (Left panel) Antisense oligonucleotides (AS) complementary to murine TACE mRNA displayed a dose-dependent (20 and 40 μ M) inhibition of endogenous TACE mRNA amounts in cultured lung explants, as demonstrated by the electrophoretic pattern of TACE competitive PCR, while both scrambled oligonucleotides (SR) at the above doses and media control (MC) did not change mouse lung TACE mRNA levels in culture. TGF- α mRNA expression was apparently not affected in lung culture regardless of TACE oligonucleotide treatment. **(Right panel)** After densitometric quantification, endogenous TACE mRNA levels in TACE oligonucleotide-treated lungs after β -actin normalization using media control as 100%. TACE antisense oligonucleotide reduced endogenous TACE mRNA levels in a dose-dependent fashion relative to media control, while TACE scrambled oligonucleotide did not significantly change the endogenous TACE mRNA amounts in cultured embryonic mouse lungs (* $P < 0.05$). **(B) (Left panel)** Western analysis of cultured mouse lungs. TACE antisense oligonucleotide at 40 μ M concentration inhibited endogenous TACE protein expression in cultured lungs relative to media control, scrambled oligonucleotide-treated, or sense oligonucleotide-treated lungs at the same concentration. **(Right panel)** Likewise, densitometric analysis of Western blot of cultured lungs showed a significantly reduced TACE protein level in lung explants cultured with TACE antisense oligonucleotide, in comparison to either TACE scrambled or sense control oligonucleotide (* $P < 0.05$). MC, media control; SR, scrambled; SE, sense; AS, antisense.

Attenuation of endogenous TACE gene expression using TACE-specific antisense oligonucleotide in embryonic lungs in culture

Since TAPI, a metalloproteinase inhibitor, is shown to inhibit embryonic lung branching morphogenesis, we decided to analyze the function of TACE as a membrane sheddase during the process of lung growth and development. While TACE gene expression takes place during early lung branching morphogenesis in culture (data not shown), the resultant biological significance on embryonic lung morphogenesis was explored using a "loss-of-function" strategy. We developed an antisense oligonucleotide to achieve efficient inhibition of endogenous TACE gene expression during lung branching morphogenesis in culture.

Inhibition of endogenous TACE gene expression using antisense oligonucleotide to TACE in lung explant culture was verified by measurement of mouse TACE mRNA amounts in cultured embryonic lungs. Competitive PCR assays were utilized to quantify the endogenous levels of TACE mRNA in lungs cultured with media control, scrambled, sense control or antisense oligonucleotide to TACE. As shown in the competitive PCR electrophoretic patterns in Fig. 2A, lungs cultured with both 20 and 40 μ M scrambled control oligonucleotide yielded levels of TACE mRNA comparable to that of media control. However, in the presence of antisense oligonucleotide to TACE, lungs demonstrated reduced amounts of

endogenous TACE mRNA in culture. While TACE mRNA levels in all assayed samples were visually compared based on the electrophoretic patterns (Fig. 2A, left panel), accurate quantification was obtained after densitometric analysis and subsequent calculation (Fig. 2A, right panel). Antisense oligonucleotide to TACE inhibited endogenous TACE mRNA levels in a concentration-dependent fashion: 20 μ M TACE antisense oligonucleotide decreased endogenous TACE level to a 53%, and 40 μ M to a further 5% of basal level ($P < 0.05$). However, none of the scrambled control oligonucleotide to TACE at either of the above concentrations significantly changed endogenous TACE mRNA expression in cultured lungs (Fig. 2A, right panel). The above results indicate that only TACE antisense oligonucleotide, not scrambled or sense (not shown) control oligonucleotide, effectively blocked endogenous TACE mRNA expression in the developing lungs in culture.

Since TACE is known to proteolytically process membrane-bound proteins including both pro-TNF- α and pro-TGF- α , in a post-translational level, we confirmed that TGF- α gene expression was not affected by TACE antisense oligonucleotide treatment. TGF- α mRNA levels were quantified in the lungs cultured with TACE oligonucleotides (Fig. 2A). Although antisense oligonucleotide to TACE efficiently attenuated TACE mRNA expression in embryonic lungs in culture, endogenous TGF- α mRNA expression was not affected by TACE antisense oligonucleotide. It seems TGF- α , a

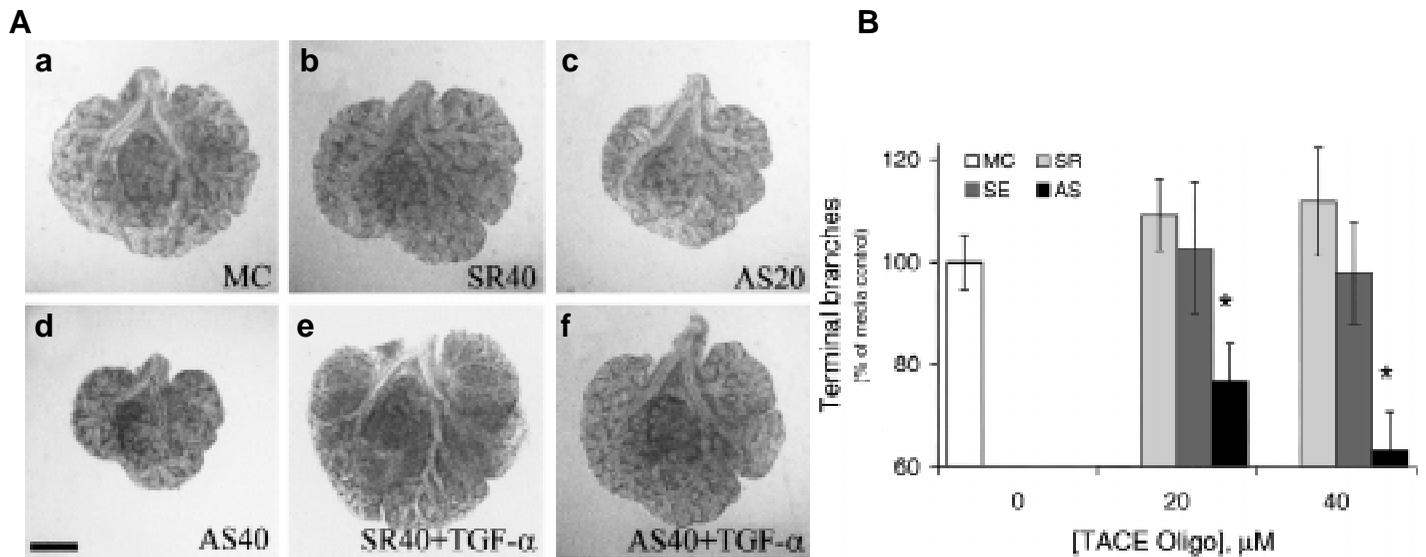


Fig. 3. Abrogation of endogenous TACE gene expression using TACE antisense oligonucleotide inhibits embryonic lung branching morphogenesis in culture. (A) Embryonic mouse lung branching morphogenesis in culture. A dose-dependent inhibition of branching morphogenesis was shown in lungs cultured with 20 (c) and 40 μM (d) antisense oligonucleotide to TACE mRNA, in comparison to either media control (a) or 40 μM scrambled oligonucleotide control (b). While addition of TGF- α (50 ng/ml) stimulated lung branching morphogenesis in scrambled oligonucleotide-treated control lung culture (e), inhibited branching in TACE antisense oligonucleotide-treated lungs was reversed in the presence of the same dose of exogenously-added TGF- α (f). Bar, 100 μm . **(B)** Antisense oligonucleotide to TACE reduced lung branching morphogenesis in a concentration-dependent manner. As determined by the number of terminal buds, TACE antisense oligonucleotide (20 and 40 μM) decreased embryonic lung branching morphogenesis in culture in a dose-dependent fashion (* $P < 0.05$), while both scrambled and sense control oligonucleotides did not alter lung branching relative to media control. MC, media control; SR, scrambled; SE, sense; AS, antisense.

protein shed by TACE, gene expression is not altered regardless of the absence of TACE due to TACE antisense oligonucleotide treatment in lung culture.

Diminished TACE protein expression in the presence of TACE antisense oligonucleotide was also shown using Western analysis on total protein lysates extracted from cultured lungs. Cultured lungs in the presence of 40 μM TACE antisense oligonucleotide resulted in inhibited level of TACE protein, in comparison to lungs with scrambled, sense oligonucleotide, or media control treatment at the same concentrations (Fig. 2B, left panel). After densitometric scanning of Western blot, endogenous TACE protein amount was reduced to a merely 13% ($P < 0.05$) of media control with addition of TACE antisense oligonucleotide at 40 μM , while either scrambled or sense TACE control oligonucleotide at the same dose did not show any inhibitory effect on endogenous TACE protein expression in cultured lungs (Fig. 2B, right panel). Thus, the TACE antisense oligonucleotide we have devised herein both significantly and specifically inhibits its corresponding TACE gene expression at both transcriptional and translational levels during embryonic lung branching morphogenesis in culture.

Abrogation of endogenous TACE gene expression inhibits lung branching morphogenesis and epithelial differentiation in culture

To facilitate the evaluation of biological function of TACE during early lung organogenesis, the TACE antisense oligonucleotide we have developed was used in the developing mouse lungs in culture. In cultured embryonic mouse lungs, an inhibition of lung branching was shown when antisense oligonucleotide to TACE at both 20 and 40 μM was added directed to culture media (Fig. 3A,

"c" & "d"), whereas parallel cultures with scrambled (b), sense (not shown) oligonucleotide did not have any inhibitory effect on lung branching morphogenesis in comparison to media control (a) lungs. After quantification of terminal branches, TACE antisense oligonucleotide-treated lung explants resulted in an oligonucleotide concentration-dependent inhibition on branching morphogenesis (Fig. 3B): antisense oligonucleotide to TACE reduced embryonic lung branching by 23% and 37% at 20 and 40 μM , respectively ($P < 0.05$). However, both scrambled (108% and 112% of media control at 20 and 40 μM , respectively) and sense (102% and 98% of media control at 20 and 40 μM , respectively) control oligonucleotide to TACE yielded unaltered levels of lung branching morphogenesis in comparison to media control (Fig. 3B).

To examine the biological impact of loss of TACE gene expression on pulmonary epithelial cell differentiation in embryonic lungs, we analyzed the expression patterns of surfactant protein-C (SP-C) and aquaporin-5 (Aqp5) in cultured lungs as differentiation markers for pre-alveolar type II and pre-alveolar type I cells, respectively (Singh *et al.*, 1985; Wuenschell *et al.*, 1996; Funaki *et al.*, 1998; Ramirez *et al.*, 2000). Proteins for both SP-C and Aqp5 were localized to the cultured embryonic mouse lungs using immunocytochemistry (Fig. 4). In cultured lungs treated with TACE scrambled control oligonucleotide, intensive immunostaining for SP-C protein was detected in peripheral airway epithelium (Fig. 4A, arrow heads), whereas both proximal epithelium (arrow) and mesenchyme were free of SP-C protein deposition. In comparison, only weak SP-C immunostaining was observed in reduced formation of terminal epithelial cells in cultured lungs in the presence of TACE antisense oligonucleotide (Fig. 4B, arrow heads), suggest-

ing a defective alveolar type II differentiation in cultured lung epithelium in the absence of normal TACE gene expression.

Pre-alveolar type I cells, as revealed by Aqp5 immunohistochemistry, was seen in the distal epithelium in cultured lungs added with TACE scrambled control oligonucleotide (Fig. 4C, arrow heads). However, only sporadic Aqp5 immunostaining was found with reduced intensity in poorly-developed terminal bronchial epithelium in lungs treated with TACE antisense oligonucleotide in culture (Fig. 4D, arrow heads), indicating an impaired alveolar type I cell differentiation in cultured lungs without TACE expression. Taken together, we conclude that both lung branching morphogenesis and concomitant epithelial cytodifferentiation were significantly inhibited in the developing lungs with deficient TACE gene expression in culture.

Exogenous addition of TGF- α reverses both inhibited branching morphogenesis and delayed epithelial cytodifferentiation in TACE antisense oligonucleotide-treated lungs in culture

We have established that TACE sheddase deficiency leads to impaired embryonic lung morphogenesis in culture. We thus reasoned that the underdeveloped lung phenotypes in culture in the presence of TACE antisense oligonucleotide may represent a lack of soluble cytokines or/and growth factors necessary for the normal lung organogenesis. Transforming growth factor- α (TGF- α) is one of the substrates known to be processed by TACE, and phenotypic defects associated with TACE null mutation are similar to those of mice lacking TGF- α (Luetteke *et al.*, 1993; Mann *et al.*, 1993; Berkowitz *et al.*, 1996; Peschon *et al.*, 1998). Since EGF, a TGF- α homologue, has been shown to stimulate lung branching morphogenesis in culture (Warburton *et al.*, 1992), we demonstrated in our preliminary studies that the addition of exogenous TGF- α also enhanced embryonic mouse lung branching morphogenesis in the explant culture (data not shown).

We further investigate whether supplementation of exogenous stimulatory factors such as TGF- α could rescue the TACE antisense oligonucleotide-treated lung culture from the inhibited lung branching morphogenesis and cytodifferentiation. As shown in Fig. 5, TGF- α (50 ng/ml), when added exogenously, stimulated lung branching morphogenesis significantly in media, TACE scramble, or sense control oligonucleotide-treated lung culture ($P < 0.05$). While lungs cultured in the presence of TACE antisense oligonucleotide branched poorly when compared with control cultures ($P < 0.05$), addition of 50 ng/ml TGF- α in culture stimulated TACE antisense oligonucleotide-treated lung branching to 106% of the number of terminal branches in lungs in plain medium ($P < 0.05$), arguing that the inhibited branching morphogenesis of TACE antisense oligonucleotide-treated lungs was overcome in the presence of soluble TGF- α added exogenously to the culture.

The biological effect of TGF- α addition to lung culture with abrogated TACE gene expression was also studied on epithelial cell differentiation during lung morphogenesis in culture. Both SP-C and Aqp5 mRNA expression were quantified using competitive PCR assays in cultured embryonic lungs (Fig. 6). While TACE antisense oligonucleotide reduced both SP-C and Aqp5 gene expression in comparison to TACE scrambled control oligonucleotide-treated lungs in culture, inhibition of both SP-C and Aqp5 mRNA expression was reversed in TACE antisense oligonucleotide-treated lungs with addition of exogenous TGF- α (Fig. 6A). In

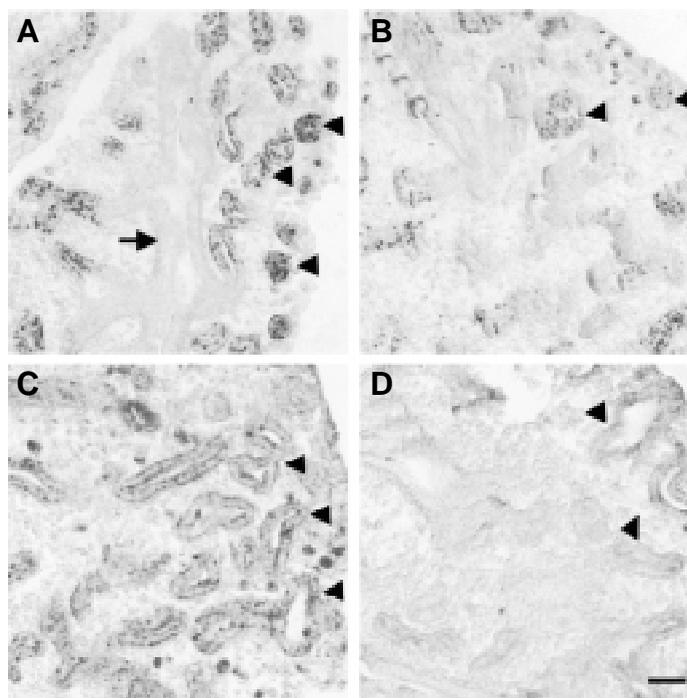


Fig. 4. Attenuated SP-C and Aqp5 immunoreactivities in cultured embryonic mouse lungs treated with TACE antisense oligonucleotide.

In TACE scrambled control oligonucleotide-treated lung culture, both SP-C (A) and Aqp5 (C) were immunolocalized in distal epithelial cells around peripheral airways, in respective pre-type II and pre-type I alveolar cells (arrow heads). In contrast, diminished immunoreactivities for both SP-C (B) and Aqp5 (D) were exhibited in TACE antisense-treated lungs in culture (arrow heads). Bar, 50 μ m.

the presence of TACE antisense oligonucleotide, both SP-C and Aqp5 mRNA levels were decreased to respective 43% and 35% of lung culture treated with TACE scrambled control oligonucleotide ($P < 0.05$) (Fig. 6B). However, addition of soluble TGF- α restored mRNA level of both SP-C and Aqp5 in TACE antisense oligonucleotide-treated lungs (93% and 90% of TACE scrambled control oligonucleotide-treated lung culture, respectively) (Fig. 6B). Therefore, TGF- α , when added in a soluble form to lung culture, could rescue the lungs from both inhibited lung branching morphogenesis and defective epithelial differentiation due to a lack of TACE gene expression in the presence of TACE antisense oligonucleotide.

Discussion

In this report, we demonstrated that TACE gene expression is essential for the normal pattern formation during lung development including branching morphogenesis and epithelial cytodifferentiation. We have shown that attenuated TACE gene expression using TACE antisense oligonucleotide resulted in both inhibitory phenotype of lung branching and reduced epithelial pre-alveolar cell differentiation in lung culture, indicating that TACE is required for optimal lung growth and development. Furthermore, we have observed that addition of TGF- α , a growth factor that stimulates lung morphogenesis, could complement the inhibited lung branching and delayed cytodifferentiation as seen in TACE antisense

oligonucleotide-treated lungs due to lack of TACE gene expression. Therefore, we have provided novel information that TACE-mediated shedding process is critical for normal lung growth and development, establishing a functional connection between TACE deficiency to a lack of stimulatory proteins necessary for lung morphogenesis such as TGF- α .

Protein ectodomain shedding represents an important and efficient strategy to regulate the activity of a variety of transmembrane proteins (Werb and Yan, 1998). The vast majority of membrane proteins can be cleaved by members of the ADAM protease family (Werb, 1997). Shedding of cytokines or their receptors can result in agonistic or antagonistic signal transductions. Upon shedding, such cytokines are distributed through the circulation and therefore act systemically, while cytokine signals may also be attenuated by soluble cytokine receptors. Therefore, protein ectodomain shedding influences the biology of the cognate growth factors and cytokines (Rose-John and Heinrich, 1994). The large number of members of the ADAM family suggests that there could be subtle substrate specificities of shedding proteases leading to differential shedding of transmembrane proteins (Black and White, 1998).

Shedding of membrane proteins is strongly induced by phorbol ester, which are known to activate protein kinase C (Arribas *et al.*, 1996; Black *et al.*, 1997; Peschon *et al.*, 1998). In addition, it has been exhibited that ectodomain shedding of transmembrane proteins including TGF- α can be induced by receptor tyrosine kinase activation and MAP kinase signaling cascades (Fan and Derynck, 1999). The identity of the responsible ectodomain-shedding proteases has been further addressed using hydroxamate based substances, which are potent inhibitors of metalloproteinases (Mohler *et al.*, 1994; Gearing *et al.*, 1994; McGeehan *et al.*, 1994). Despite the fact that the cleavage sites of the membrane proteins lack sequence homologies, the common features of the shedding processes indicated that the proteases involved were similar if not identical (Hooper *et al.*, 1997).

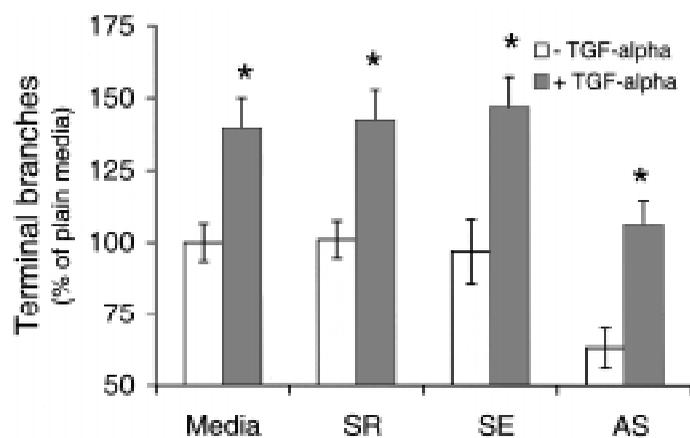


Fig. 5. Exogenous addition of TGF- α rescues the TACE antisense oligonucleotide-treated lungs from inhibited branching morphogenesis in culture. While exogenously-added TGF- α stimulated lung branching morphogenesis in media, scrambled oligonucleotide-, or sense oligonucleotide- control lungs in culture (* $P < 0.05$), addition of TGF- α also increased the branching of TACE antisense oligonucleotide-treated lungs in culture (* $P < 0.05$). SR, scrambled; SE, sense; AS, antisense.

The biological importance of TACE was underlined by the perinatal lethal phenotype of mice homozygous of mutated TACE genes (Peschon *et al.*, 1998). As mice lacking TNF or its receptor are overtly normal, it was suspected that TACE has enzymatic activities in addition to release of TNF- α (Peschon *et al.*, 1998). Mice lacking TACE show a similar phenotype to animals defective of TGF- α or its cognate receptor, EGFR, suggesting the involvement of TACE not only in pro-TNF- α shedding but also in membrane cleavage of EGF-like ligand precursors (Peschon *et al.*, 1998; Luetteke *et al.*, 1999). Furthermore, other EGFR-binding ligands, such as EGF, heparin binding EGF-like growth factor (HB-EGF), amphiregulin, are initially synthesized as transmembrane precursors and are proteolytically cleaved by metalloproteinases to release the mature growth factors in an autocrine or paracrine manner (Massagué and Pandiella, 1993). Metalloproteinase-mediated membrane proteolysis of EGF family molecules may thus represent a general mechanism to activate EGFR signal transduction (Leserer *et al.*, 2000).

EGF family members are important epithelial mitogens and their receptor, EGFR, is considered a prototype tyrosine kinase receptor (Cohen *et al.*, 1980). EGFR inactivation by homologous recombination resulted in epithelial immaturity in organs undergoing branching morphogenesis during development, such as lung, kidney, mammary gland, pancreas, and prostate (Miettinen *et al.*, 1995). The respiratory failure of the EGFR null mice arises from impaired branching of the pulmonary alveolar tree and leads to reduced surface for gas exchange. The mouse lung phenotype without functional EGFR bears similarity to respiratory distress syndrome and bronchopulmonary dysplasia, the most common complications of prematurity in humans (Miettinen *et al.*, 1997). Further evidence has shown that EGF enhances lung maturation in fetal rabbits and stimulates lung branching morphogenesis in fetal mouse lung culture (Catterton *et al.*, 1979; Warburton *et al.*, 1992). It is likely that EGF-like signals modulate embryonic lung proliferation and differentiation.

In the current study, we have shown that TGF- α , like EGF, stimulates lung branching morphogenesis in embryonic mouse lung culture, indicating that TGF- α signals, mediated through EGFR, is permissive for lung growth and development. Several lines of experimental evidence have suggested that TGF- α plays an functional role during normal lung development and pulmonary pathogenesis. TGF- α and EGFR are expressed in the epithelium of the developing lungs (Strandjord *et al.*, 1995). In humans, elevated TGF- α protein levels are found in acute and chronic lung injury, and TGF- α levels from bronchoalveolar lavage fluid increase in patients with acute respiratory distress syndrome (Madtes *et al.*, 1998). Transgenic mice expressing TGF- α in type II pneumocytes under control of the lung-specific surfactant protein-C (SP-C) promoter develop pulmonary fibrosis and marked air-space hypoplasia, while TGF- α deficiency can reduce fibrogenesis in TGF- α null mice (Korfhagen *et al.*, 1994; Madtes *et al.*, 1999). In this context, growing evidence points to the TGF- α as a key regulator of normal and abnormal lung development.

TACE-mediated membrane protein shedding constitutes an important cellular regulatory mechanism, and enzymatic release of not only TNF- α but also TGF- α is efficiently blocked in cells with inactivated TACE (Peschon *et al.*, 1998). Based on the previous findings that EGF family ligands precursors are proteolytically cleaved by metalloproteinases and that TGF- α is a key morphogen

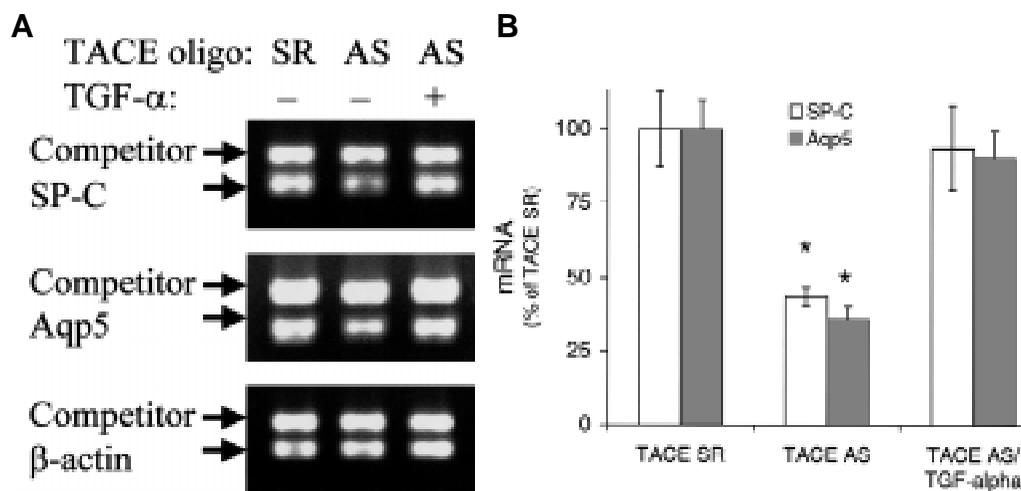


Fig. 6: Reversal of down-regulated SP-C and Aqp5 mRNA expression by TGF- α in cultured lungs with deficient TACE gene expression. (A) In comparison to lungs cultured in the presence of TACE scrambled control oligonucleotide, both SP-C and Aqp5 mRNA expression in embryonic lungs were reduced by treatment of TACE antisense oligonucleotide in culture, as shown by electrophoretic patterns of respective SP-C and Aqp5 competitive PCR. However, addition of soluble TGF- α restored mRNA expression of both SP-C and Aqp5 in TACE antisense oligonucleotide-treated lungs to levels comparable to those lungs treated with TACE scrambled control oligonucleotide. (B) Densitometric quantification

confirmed that exogenous treatment of TGF- α could reverse the inhibited mRNA expression of both SP-C and Aqp5 in the presence of TACE antisense oligonucleotide in embryonic lung culture (* $P < 0.05$). SR, scrambled; SE, sense; AS, antisense.

for the lung development (Miettinen, 1997; Leserer *et al.*, 2000), it is conceivable that the hypoplastic phenotype we have seen in the cultured lungs with TACE inactivation, using either TAPI or TACE antisense oligonucleotide, is, at least partially, due to a lack of soluble TGF- α in lung culture. This hypothesis is further supported by our observation that addition of exogenous TGF- α into the lung culture medium rescued TACE-deficient lungs from inhibition of both branching morphogenesis and epithelial cytodifferentiation. While TGF- α , like EGF, is a known positive factor for lung morphogenesis, the current findings support the notion that a lack of diffusible TGF- α ligands may contribute to the inhibited lung growth and development in cultured lungs with attenuated TACE gene activity. The present experimental results suggest that the TACE protein exerts its biological effect through altering availability of soluble molecules including TGF- α , thereby regulating lung growth and development.

TACE appears to be involved in the proteolytic release of multiple membrane-bound proteins including cytokines, growth factors, and their receptors (Müllberg *et al.*, 2000). Of them, many are important intercellular regulators that have important roles during mammalian development (Schlöndorff and Blobel, 1999). In the current report, we have presented evidence that EGF-like signals including TGF- α may be implicated in the TACE-mediated biological mechanism in the developing lungs in culture. However, recent evidence has accumulated for an expanded role for TACE in the proteolytic process of a repertoire of cell surface proteins with growing numbers (Müllberg *et al.*, 2000), it is possible that the lung hypoplasia in the absence of TACE metalloproteinase activity is due to a deficiency of multiple intercellular regulators permissive for normal lung morphogenesis including that of TGF- α .

Analysis of mice carrying targeted disruptions of TACE gene indicates that TACE has essential role in mammalian development (Peschon *et al.*, 1998). We have demonstrated a pulmonary hypoplasia in embryonic mice lacking TACE gene expression (Zhao *et al.*, 2001). The present work demonstrates that a lack of TACE activity, using either TACE inhibitor TAPI or TACE-specific antisense oligonucleotide in mouse lung culture, results in not only inhibition of lung branching morphogenesis but also reduced expression of both Aqp5 and SP-C genes, suggesting an underde-

veloped lung phenotype in the absence of TACE. Thus, we have provided direct evidence that TACE deficiency leads to abnormal lung branching morphogenesis and cytodifferentiation during embryonic lung morphogenesis in culture. It seems that TACE gene expression is necessary for the normal pulmonary pattern formation during lung organogenesis.

Although the results of this study has suggested the functional importance of TACE during lung growth and development, the regulation of expression of ADAM family members including TACE is poorly understood. Physiological stimuli such as acetylcholine and lipopolysaccharide, or synthetic compounds, such as phorbol esters, dramatically increase the release of numerous shed proteins (Hooper *et al.*, 1997). To elucidate the general mechanisms that regulate ectodomain shedding, TGF- α cleavage is found to be induced by fibroblast growth factor or platelet-derived growth factors and is critically dependent on the ERK/MAP kinase pathway (Fan and Derynck, 1999). On the other hand, TIMP-3, but not TIMP-1, 2, or 4, is a potent inhibitor of TACE activity (Amour *et al.*, 1998). Since we have shown herein that TACE-mediated shedding plays a functional role during lung branching morphogenesis and cytodifferentiation, interpreting the regulatory mechanism of TACE catalytic activity may further our understanding of the biological significance of TACE in the developing lung tissue.

In summary, we have shown that TACE plays a key regulatory mechanism during lung branching morphogenesis and epithelial cell differentiation, probably through altering the availability of soluble proteins necessary for normal lung growth and development. Even though the complete signaling mechanism for TACE is not fully understood, TACE-mediated protein ectodomain shedding appears to be indispensable for normal lung organogenesis.

Materials and Methods

TACE antisense oligodeoxynucleotide

We chose a 17-bp TACE antisense oligonucleotide, 5'-CCG CCT CAT GTT CCC GT-3', to flank the AUG initiation codon of TACE mRNA (Black *et al.*, 1997), a site known empirically to be most effective in inhibiting translation of target mRNA (Marcus-Sekura, 1988). Two same-length negative control oligonucleotides were also designed to ensure the sequence specificity of the TACE antisense oligonucleotide: (i) a randomly scrambled oligonucleotide

with the same base composition to antisense sequence; and (ii) the corresponding sense oligonucleotide. TACE antisense, scrambled, and sense oligonucleotides were screened for their uniqueness in the GenBank data base, with particular attention to lack of homology to other known genes in the ADAM family of metalloproteinases.

TACE oligonucleotides were synthesized with phosphorothioate modification to render nuclease resistance in culture (Sharma and Narayanan, 1995). Purified TACE oligonucleotides were reconstituted in sterile water and their concentrations were determined by optical density at OD₂₆₀ (USC MicroChemical Core Facility, Los Angeles, CA).

Embryonic lung explant culture

Timed-pregnant Swiss Webster female mice were obtained from Simonsen Laboratories (Gilroy, CA). Uterus were removed at gestational day 11.5 (E11.5, day of vaginal plug is defined as E0). Lung primordia were gently dissected from embryos by microdissection under sterile condition, using a dissecting microscope and jewelers forceps.

Early mouse embryonic lung explants (E11.5) were cultured in serum-free BGJb medium (Gibco, Grand Island, NY) using organ culture dishes (Falcon, Lincoln Park, NJ), as we have previously described in detail (Zhao *et al.*, 1996). TACE oligonucleotide, TAPI-1 (Peptides International, Louisville, KY), and/or TGF- α ligand (R&D Systems, Minneapolis, MN) were added exogenously to the culture media to desirable concentrations. The lungs were cultured for four days with change of media every two days. Cultured lungs were harvested for quantification of terminal branches, RNA extraction, Western analysis, and/or immunohistochemistry.

Evaluation of lung morphology and branching morphogenesis

Since branching morphogenesis *per se* is the key bioassay read-out for evaluating the functional role of TACE during early lung organogenesis, three independent measurements of branching morphogenesis were devised: (i) the number of airway generations from the trachea to the most distal branch of the longest visible airway; (ii) the number of air sacs visible around the periphery of the lung explants; and (iii) computerized pattern recognition analysis of the number of individual terminal respiratory units in the whole explant. Cultured lung explants were captured as computerized images under microscope using a digital CCD camera (Polaroid, Cambridge, MA). Terminal branches were defined as the most distal branches of each airway generation (Warburton *et al.*, 1992). These analyses were performed on whole-mounts of lung explants without knowledge of the assay conditions, using transillumination to visualize structures and photomicroscopy to record permanent images. Paraffin-embedded sections of cultured lung tissue after fixation were also examined to verify the counting of terminal branching.

RNA extraction, reverse transcription and competitive PCR assays

Total RNA from cultured lung explants was extracted by guanidinium thiocyanate following homogenization as we have documented elsewhere (Zhao *et al.*, 1996). Extracted total RNA was immediately reverse-transcribed by incubating at 37°C for 1 h in the presence of ribonuclease inhibitor, oligo-d(T)₁₂₋₁₈, and M-MLV reverse transcriptase (Gibco BRL). The resultant cDNA products were used for competitive PCR quantification.

Competitive PCR methodology for specific mRNA quantification of pulmonary genes has been described previously (Zhao *et al.*, 1996). Briefly, a set of primers were designed for mouse TACE to amplify a 326 bp cDNA fragment (Black *et al.*, 1997). To generate competitor cDNA for TACE competitive PCR assay, the above desired primer sequences were engineered into a heterologous DNA fragment using same strategy as we documented earlier (Zhao *et al.*, 1996). Consequently, both TACE cDNA and TACE competitor utilize the same set of primers in the TACE competitive PCR. The TACE competitor was 430 bp in length. Both TACE and its competitor PCR products were subsequently DNA sequenced to verify their identities. Competitive PCR assay for TNF- α was developed in a similar manner as that for TACE.

PCR amplification was carried out using a modification of a previously described assay for TGF- β type II receptor (Zhao *et al.*, 1996). Reverse-transcribed samples derived from 20-50 ng total RNA were added to a PCR

reaction mixture containing a known amount of competitor to achieve a total volume of 50 μ l. β -actin competitive PCR as an internal control was performed in parallel on the same assayed samples. As a negative control for genomic DNA, non-reverse-transcribed total RNA was also included in the competitive PCR assays.

Immunohistochemistry

Cultured mouse lungs were fixed in 4% paraformaldehyde for 2-3 h at room temperature and immediately embedded into paraffin. Lung specimens were sectioned to 5 μ m and mounted onto HistoGrip-coated microscopic slides (Baxter, Deerfield, IL). Both SP-C and Aqp5 antibodies (affinity purified goat polyclonal IgG) were used at concentrations according to the manufacturer's recommendations (Santa Cruz Biotech., Santa Cruz, CA). Biotinylated secondary antibody and streptavidin-peroxidase conjugate were used to detect tethered antibody (Zymed, South San Francisco, CA). The peroxidase reaction products were red-colored with aminoethyl carbazole. Normal goat IgG, bovine serum albumin, and water were run in parallel slides as negative controls.

Western blot analysis

Cultured lung explants were lysed in a SDS buffer containing protease inhibitors (Shi *et al.*, 1999). Total protein concentration in each assayed sample was determined using the Micro BCA Protein Assay Reagent kit (Pierce, Rockland, IL). Equal amounts of total protein from each conditioned sample were prepared for chemiluminescent Western analysis (Roche, Indianapolis, IN) as reported earlier (Zhao *et al.*, 2000). A goat polyclonal anti-TACE antibody was used at a dilution of 1:500 (Santa Cruz Biotech). β -actin immunoblotting was also performed to ensure equal loading in all assayed lanes.

Statistics

Groups of ten or more lungs were collected for each experiment. Data are presented as means \pm SD. To test for statistically significant differences, the Student's *t* test was used. A value of *P* < 0.05 was considered significant.

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