質問 10　物が見えづらい状態と音が聞こえづらい状態のどちらが想像しやすいですか。

この質問は健聴者の難聴に対する認識の上で、聞こえにくさのイメージのしづらさに何らかの関係があるのではないかと考え、設けた。

表 16

| 想像しやすい項目 | 人数 | ﾊﾟｰｾﾝﾄ |
|---|---|---|
| 音が聞こえづらい | 75 | 31.4 |
| 物が見えづらい | 164 | 68.6 |
| 合計 | 239 | 100.0 |

「物が見えづらい」方が、「音が聞こえづらい」よりも想像しやすいと答えた人の割合は 68.6% であった。この結果から、「音が聞こえづらい状態」が一般的にはイメージしにくいと考えられる。その背景には比較した「物が見えづらい状態」は、視力低下という身近な現象に置き換えて想像しやすかったのではないかと思われる。

聴力の低下も視力の低下と同じく老化に伴って発生していくものではあるが、アンケートの対象者が大学生という若い集団であったので、より「音が聞こえづらい状態」を想像しやすい項目に選ぶ傾向につながったのではないかと考えられる。

C. 3. 難聴者の経験や悩み

C. 3. 1. 面接調査から

聴覚障害を持つ F 氏 (19 歳・男性) に面接調査を行った。聴覚障害を持つ身での経験や悩んだこと、思いなど語ってもらった事を以下に記述する。

● F 氏について

先天性難聴で、難聴以外にも病気を持って産まれてきた。聴力は健聴者と難聴者の境目くらい。普段は補聴器を装用して生活している。大学まで全て通常学校で過ごしていた。

● 普段の生活で工夫していること

・ 補聴器の集音する側が、友達や話す人側になるように立ち位置や座り位置、顔の向きを変える。
・ 学食などざわつきがある時等は聞き取りにくくなるため、話している人や言葉を取り逃がさないように、目線を話している人 (話したかもしれない人) の方に向けて口もとを見るようにしている。

● 聞こえの面で困ること

・ 補聴器を装用しているため、机や椅子等を動かす音はやはりうるさく感じる事はある。しかし、うるささの程度はわからないが、それは健聴者も同じ事であり、我慢することは普通だと思う。聞き分けはできる方なので、もっと重度の人には大変な事かもしれない。
・ 会議や発表の時は一人一人で話すから平気だが、話し合いの場はいろんな人が秩序なく話すから途中でついていけなくなってしまう事がある (誰が発言するか予測できないため、顔を向けたりする対象がかわるがわるになってしまう) 。

● 対人関係で困ったこと

・ 小学校の時は耳が聞こえにくいという事でからかわれた。中学時代もいざこざはあったが、高校に入ってからは周囲も理解をしてくれるようになった。
・ 話している人の顔や「今、何か言ったのか

な？」という人の顔をみる習慣があるため、「じっと見ているなよ」と言われてしまうこともあった。

・ 補聴器をしていて集音できている時は聞こえるし発音もできる方なので、たまたま方向が悪くて聞こえなかった時に「無視した」「聞こえているくせに」と誤解されてしまう事もあった。

・ 聞き取りにくい時は何度も聞き返してしまう事があるが、その時に面倒に思われてしまうことがあった。相手を理解したくて聞き返しをするのだが、その想いが伝わらないことがたびたびあった。

C. 3. 2. 施設訪問から

信州大学医学部耳鼻咽喉科　宇佐美真一教授を訪ねお話を聞き、また、付属病院人工内耳センターを見学した。

● 信州大学医学部付属病院人工内耳センター

重度の聴覚障害を持つ患児とその保護者に対して、人工内耳に関する情報やガイダンス、手術前の検査、手術後の人工内耳機器の調整や管理、聴能言語ハビリテーションなどを行っている。また、患児の通う聾学校 (特別支援学校) や通常学校とも連携して成長を支える環境づくりをしている。

実際に人工内耳を装用している幼児との活動を見学したが、遊びの中で正しい発音の練習をさせたり、聴神経を刺激させたりしていた。また、活動中の子どもの発する声の大きさをもとに人工内耳の出力の調整も行っていた。見学した子どもたちは言語獲得前に人工内耳を装用し、人工内耳センターのような環境の整ったところで訓練をしていたため、話すことも聞き取りも上手く、活動の様子は普通の幼児教室のようで大変驚いた。そのくらい、早期の人工内耳の装用は効果的なものであると感じた。

● 支援員の方の話

人工内耳センター内の難聴児支援センターの支援員の方から伺ったことを以下に記述する。

・ 難聴を抱える子どもの保護者の方の想い

人工内耳センターに積極的に通う保護者の方の願いは、通常学校に子どもを通わせる事なのかと思い、支援員の方に質問したところ、必ずしもそうではない、と返ってきた。もちろん健聴児と同じように聞こえ、同じような言語能力が身に着くことは願いであり、喜びではあるが、その時の発達状況に応じて、子に合った環境や学校で学ばせたいという想いもあるそうだ。

その反面、幼稚園児の保護者で、聾学校 (特別支援学校) の小学部に見学に行ったが、手話を使っている様子を見て、通常学校に在籍させて今ある言語能力を高めていきたいという考えを持つ家庭もあったようだ。

・ 難聴児の学校選択について

通常学校に通うか特別支援学校に通うかという決断の要素には学校の設備的な環境や教師の難聴への知識だけではない。子どもどうしの学びあいの面でもある。

通常学校に通うメリットは言語能力を高める面である。聾学校のメリットは設備や教師の知識が整っている面である。しかし、それ以外に難聴児にかかわらず成長の過程で大切なことがある。それは、子どもが同世代のモ

デルを見ることである。特別支援学校に通えば同じような障害を抱えるモデルを見ることによって成長面で良い面もあるかもしれないが、通常学校に通った方がモデル数はたくさん見ることができる。

これらのことから、この人工内耳センターに通っている子どもたちは週の何日かは特別支援学校に通い、残りの日は通常学校・幼稚園に通っているということであった。

## D. 考察

### D. 1. 難聴者と医療

近年、新生児聴覚スクリーニングが普及してきたことにより、早期に難聴を発見できるようになってきた。そのため、幼児期の早い段階からその子どもにあった補聴器や人工内耳を装用する手段を選ぶことが可能になり、聴力の習得のみならず言語の習得もかなりのレベルまで可能になった。宇佐美真一教授がセンター長を務める「信州大学付属人工内耳センター」では、新生児聴覚スクリーニングから保護者や患児の支援、人工内耳のマッピング、(リ) ハビリテーションまで総括的に管理・支援している。このような医療施設は増えつつあるが、まだまだ一般的に利用できるまでは広がってはいない。いまだそのような医療施設の整っていない都道府県も数あるのが現状である。近くにそのような医療施設がないために、手段の選択肢を狭めてしまうこともあると推測されこのような取り組みの整備が全国的に必要であると感じた。

医療の発達により難聴者の聴力レベルや言語獲得の能力は飛躍的に向上し大変素晴らしい成果である。しかし、一方で健聴者には難聴を抱えている人や抱えている事がわかりにくくなってきた。このことは、補聴器の効果の限度である集音する角度や「はっきり聞こえない」という症状に対応しきれない面で「聞こえているはずなのに無視された」など誤解を受けやすくなっていることも難聴者の経験 (F 氏との面接調査) から伺える事実である。医療によって難聴者の従来の壁であった聴覚や言語そのものの QOL は目覚ましく向上してきているが、人々 (健聴者) からの認識はまだまだ十分とは言えず、対人関係等の社会的な面での QOL は医療の発達だけでは解決しがたい問題だと考えられる。

### D. 2. 難聴者と教育

医療の発達に伴う聴力レベルや言語習得レベルの向上により、難聴を抱える子どもたちの学習の場は、近年、聾学校 (特別支援学校) 以外にも広がっている。人工内耳センターの支援員の方も話されていたように、難聴児が健聴児と共に生活を送ることは言語能力の維持や向上の面で有益な事である。また、在籍児童数は通常学校の方が聾学校よりも圧倒的に多いため、難聴児は同世代の子どもとより多く接することができる。これは健聴者側にも同じ事が言える。近年、「インクルージョン教育」という考えが注目されている。障害を持つ・持たないに関わらず、排除されるおそれのある多様なニーズをもつ全ての子どもを対象に通常学校の現場でそれぞれのニーズへ対応していくという考えのものである。この考えは排除される恐れのある子どももそうでない子どもも皆一緒に学ぶことによって対人関係を学ぶ機会や、障害を持つ子どもに関してはその障害についての認識を深める等の機会になると考えられる。難聴児に置き換えると、通常学校に通う事によって、周りの子どもたちも学校生活や遊びの中で自ずと障害に

関する認識が高まり工夫を見出すことが出来るようになる機会になり得ると考えられる。このことは学校就学世代である人格形成期の子どもたちにとって、とても意味のあることであると思われる。

D. 3. 難聴者と健聴者の意識

特別講義 I の感想から、難聴の原因について今まで知らなかったという意見や、人工内耳というものの存在を初めて知ったという意見、意外とたくさん難聴者がいることに驚いたという意見が多かったことから健聴者にとって、「難聴」そのもの自体が身近な存在でないということが明らかになった。難聴や難聴者に対する認識を高めるには、難聴や難聴者を身近に感じる事が大切であると考えられる。このことから、身近に感じている者とそうでない者の差は、おそらく難聴について知る機会が無かったからではないかと考えられた。本研究で行ったアンケート調査から、「物が見えにくい状態 (視覚障害)」の方が聴覚障害の症状よりも想像しやすい (表 16) という結果が得られた。実際に視力の低い者が多く、視覚の方が想像しやすいのではないかと考えられる。また、本調査では、約 6 割の人が補聴器の効果を正しく認識していないことが明らかになった。補聴器には音を大きくする効果はあるが、音を鮮明にする働きはない。しかし、表 12 で示したように、不正解である「音を鮮明にする効果」「音を鮮明にし、大きくする効果」を選択した者が全体の 61.1% を占めた。F 氏との面接調査でも述べたように、補聴器を装用していれば聞こえるようになるという誤った認識をもち、このことが難聴者の対人関係での QOL に深くかかわっていると思われる。この結果は、健聴者の補聴器に関する認識がいかに低いものであるか、またそれが難聴者の悩みに深く関わっているのかが、よく表されたものであると考えられる。さらに、健聴者と難聴者が通常学校で共に学ぶ事について、健聴者に「健聴者の立場」と「難聴者の立場」の両方の立場になったつもりで回答してもらったが、「健聴者の立場」では賛成と答える者が 6 割と多かった。しかし、「難聴者の立場」ではわからないと答えた者が 5 割弱にまで達した。「賛成」の理由の内訳では「差別はよくない」「一緒にいることで健聴者が学ぶ事があると思う」というものが多かった。「わからない」の理由の内訳では「障害の程度による」「気を遣わせてしまうと思うと迷う」というものが多く見られた。この結果は、健聴者としての立場では一緒に難聴者と学ぶ事に関して肯定的だが、いざ、難聴者の立場になると消極的になる者が多いということであり、おそらく健聴者は難聴者と共に過ごすことに関しては特に気を遣うことはないが、難聴者の立場ではどういう事に困る可能性があるのかという事に対する認識があまりなく、想像しにくいというのではないかと考えられた。健聴者が持つ難聴という障害の状態に対する認識の程度はあまり高くないと考えられる。

健聴者に対するアンケートにおいて、これまで難聴者と出会いあったか、ないかという分類をしてみると、出会いがない人はある人と比較して、難聴者を「聞くことが出来ない」、「可哀想」、「手話を使う」と思う人が多かった (表 8, 9)。出会いのあった者はある程度交流をしていたり、人工内耳や補聴器の存在を知っていたりしたためにこのような結果になったと推測される。特に、「聞くことが出来ない」と「手話を使う」というイメージは関連

があり、聞くことができないから手話を使う、という連想がされやすいのではないかと考えられる。現実に手話を使う人はたくさんいるものの、人工内耳や補聴器を幼いころから装用していた者は手話を使わない事も多く、そのイメージから「コミュニケーションがとりにくい」と思われてしまうもあると予想される。このことは難聴者にとって対人関係でのストレスになり得ると推測される。同様に、難聴の症状に対するイメージでも難聴者との出会いの経験の有無で有意差がみられた (表10, 11)。「とぎれて聞こえる」と「雑音が入って聞こえる」の症状の認識が著しく低かった。その認識が著しく低く、難聴者との出会いの経験がある者ほどそれらの症状への認識が高い傾向になった。上記したことから、難聴者との出会いの経験がある者の方が、難聴や難聴者に関する認識が高い事が推測される。

D. 4.　総合考察

本研究では、社会・教育という面から難聴者と健聴者の認識や現状を把握し、難聴者のQOL を向上させるためには例えば学校教育でどのような働きかけができるか、学校という場を難聴者と健聴者の認識の差を埋める場としてどのように活用していけるのかを考察することを目的とした。

難聴者の抱える悩みや想いと、健聴者の認識の差はどこにあるのか、差が出来てしまう原因はどこにあるのか、どうしたらその差を埋める事が出来るのか。難聴者を取り巻く様々な要素について調査研究を進めると、難聴者の QOL を高める視点には様々なもの(医療、教育、福祉・移行支援) があり、それぞれがそれぞれの視点から難聴者の QOL を高めているが、最終的に QOL を高める根本的な要素は「人」である。人々の認識が医療を高めたり、教育方法を考えたり、福祉・支援を充実させようとすることも事実である。また、難聴者に関わる健聴者 (学校等での友人や、社会生活上での接点を持つ全ての人) が難聴者や難聴への理解があると難聴者にとって対人関係の面でも充実し得る。難聴者にとってよりよい社会を作り出すためには、難聴者の悩みと健聴者の認識の差を埋めていく事が必須である。教育の面で考えると、学校を難聴者の悩みと健聴者の認識の差を埋める場としてもっと活用できるであろうと考えられる。小・中・高等学校のような学校において、人格形成期である就学世代に難聴を抱える子どもたちと健聴児を一緒に学ばせる事が重要であろう。子どもたちは人格形成期にいろんなモデルを見たり接したりする事で様々な事に理解をしていく。最初は「知らなかった人・事」「身近でなかった人・事」であっても、人格形成期にある子どもたちはだんだんと日常化していくのである。この日常化とは、単なる「慣れ」というものではなく、遊びや生活での経験を通して障害自体に対する認識やそれを抱える人に対する認識が自然に高まることによって身近な事とされるという意味である。人格形成期に様々な出会いをすることは、大人になってから同じような出会いがあった時にすんなりと関係を築く事が可能であると考えられる。学校とはそのような「出会い」を経験させる場であり、その「出会い」を有意義なものにさせるのは教職員をはじめとする学校組織である。特に、学校組織の中で養護教諭はすべての児童生徒の心と身体の健康を守る任務を負っており、「人」の融合・調和を図る点で「出会い」を難聴児と健聴児双方にとって有意義なものとする為のサポー

トができると考えられる。そのためには難聴を抱える人々の背景にある様々な要素を理解し、その様々な要素（機関）の環に入り連携していく事も必要である。それと同時に、学校の機関の中で難聴児と健聴児が共に楽しく活躍でき、学びあえるような学校生活や行事等をコーディネートする役割も担う必要がある。

本研究により、健聴者は難聴という症状をあまり理解できていないということが明らかになり、また、理解しづらいのではないかと考えられる。このような点から、やはり、難聴ということを理解する前に聴覚の仕組みについての理解を深める必要があると考えられる。正常な状態の聴覚、そしてその病態、難聴という順で理解をすすめ、さらに難聴を補う補聴器や人工内耳の仕組みなどを理解してもらうような教育を行えば、補聴器や人工内耳が依然完璧なものではなく健聴者の理解や配慮も必要であるということにもつながるのではないかと思われる。また、近年、イヤホンを装着して大音量で長時間音楽を聴く風景をよく目にするが、聴覚の仕組みを理解しておけば、このようなことによる後天的な難聴の予防にもつながるのではないかと考えられる。

現在、学校現場では難聴児の受け入れ態勢、周囲の子どもたちへの教育、教職員の知識の向上等、支援態勢を整えている最中であり、課題はたくさん残っているものの「モノ」および「ヒト」による QOL 向上にむけた取り組みが必要である.

**G. 研究発表**

なし

**H. 知的財産権の出願・登録状況**

なし

## Ⅲ．研究成果の刊行に関する一覧表

雑誌

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# Salicylate-induced translocation of prestin having mutation in the GTSRH sequence to the plasma membrane

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ABSTRACT

**Prestin is a key molecule for mammalian hearing. The present study investigated changes in characteristics of prestin by culturing prestin-transfected cells with salicylate, an antagonist of prestin. As a result, the plasma membrane localization of prestin bearing a mutation in the GTSRH sequence, which normally accumulates in the cytoplasm, was recovered. Moreover, the nonlinear capacitance of the majority of the mutants, which is a signature of prestin activity, was also recovered. Thus, the present study discovered a new effect of salicylate on prestin, namely, the promotion of the plasma membrane expression of prestin mutants in an active state.**


## 1. Introduction

The motor protein prestin in the plasma membrane of cochlear outer hair cells (OHCs) is believed to be the origin of their electromotility [1]. So far, several characteristics of prestin have been clarified by introduction of mutations into prestin [2]. Mutations in membrane proteins sometimes cause the accumulation of these proteins in the cytoplasm. It has been reported that when the cells expressing such accumulated mutants were cultured with a pharmacological chaperone, which is a cell membrane-permeable molecule with high affinity for these mutants, the chaperone bound to them and promoted their transport to the plasma membrane in an active state [3–7]. Salicylate, which is known as an antagonist of prestin, is thought to have cell membrane permeability with high affinity for prestin [8,9]. Thus, salicylate was considered to be a candidate molecule to work as a pharmacological chaperone for prestin. In the present study, the aim was to investigate whether or not salicylate has the ability to promote the plasma membrane expression of prestin mutants accumulated in the cytoplasm.

## 2. Materials and methods

### 2.1. Prestin mutants

Our previous study showed that mutations in the GTSRH sequence at positions 127–131 of prestin caused a decrease in nonlinear capacitance (NLC), which is a signature of prestin activity [10]. Such decrease may be due to the accumulation of prestin in the cytoplasm. Thus, the present study used the prestin mutants created in our previous study, namely, G127A, T128A, S129A, R130A, H131A and S129T. These mutants were engineered to be expressed in HEK293 cells by transfection. As the prestin genes were co-transfected with green fluorescent protein (GFP) gene into the cells, transfected cells were selected by GFP observation.

*Abbreviations:* OHC, outer hair cell; NLC, nonlinear capacitance; GFP, green fluorescent protein; WT, wild-type; WGA, wheat germ agglutinin

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### 2.2. Incubation of transfected cells with salicylate

Transfected cells were cultured with salicylate, which is generally known to have the ability to suppress NLC as an antagonist of prestin, to confirm another effect of salicylate as a pharmacological chaperon for prestin. It was reported that, in the patch-clamp recording, 10 mM salicylate around the cells was required for almost complete suppression of NLC, which might be realized by the binding of salicylate with prestin in the plasma membrane [9]. Thus, for the binding of salicylate with prestin, at least 10 mM salicylate was considered to be necessary. Although salicylate possibly affects the cell viability, it has been reported that more than 85% of HEK293 cells were able to survive in the presence of up to 10 mM sodium salicylate [11]. In the present study, the cells were cultured for 24-36 hours in growth medium with sodium salicylate at a concentration of 10 mM from 12 hours after transfection. After such incubation, the cells were used in experiments. The cells cultured without salicylate were employed as control samples. Samples of the cells expressing wild-type (WT) prestin and its mutants which were cultured with 10 mM salicylate were termed WT prestin+Sal, G127A+Sal, T128A+Sal, S129A+Sal, R130A+Sal, H131A+Sal and S129T+Sal.

### 2.3. Confirmation of the localization of prestin in transfected cells

The localization of prestin in the cells was assessed by immunofluorescence staining with anti-FLAG antibody, TRITC-conjugated anti-mouse IgG antibody and wheat germ agglutinin (WGA)-Alexa Fluor 633 conjugate as described in our previous study [12]. In the present study, several tens of transfected cells were observed for

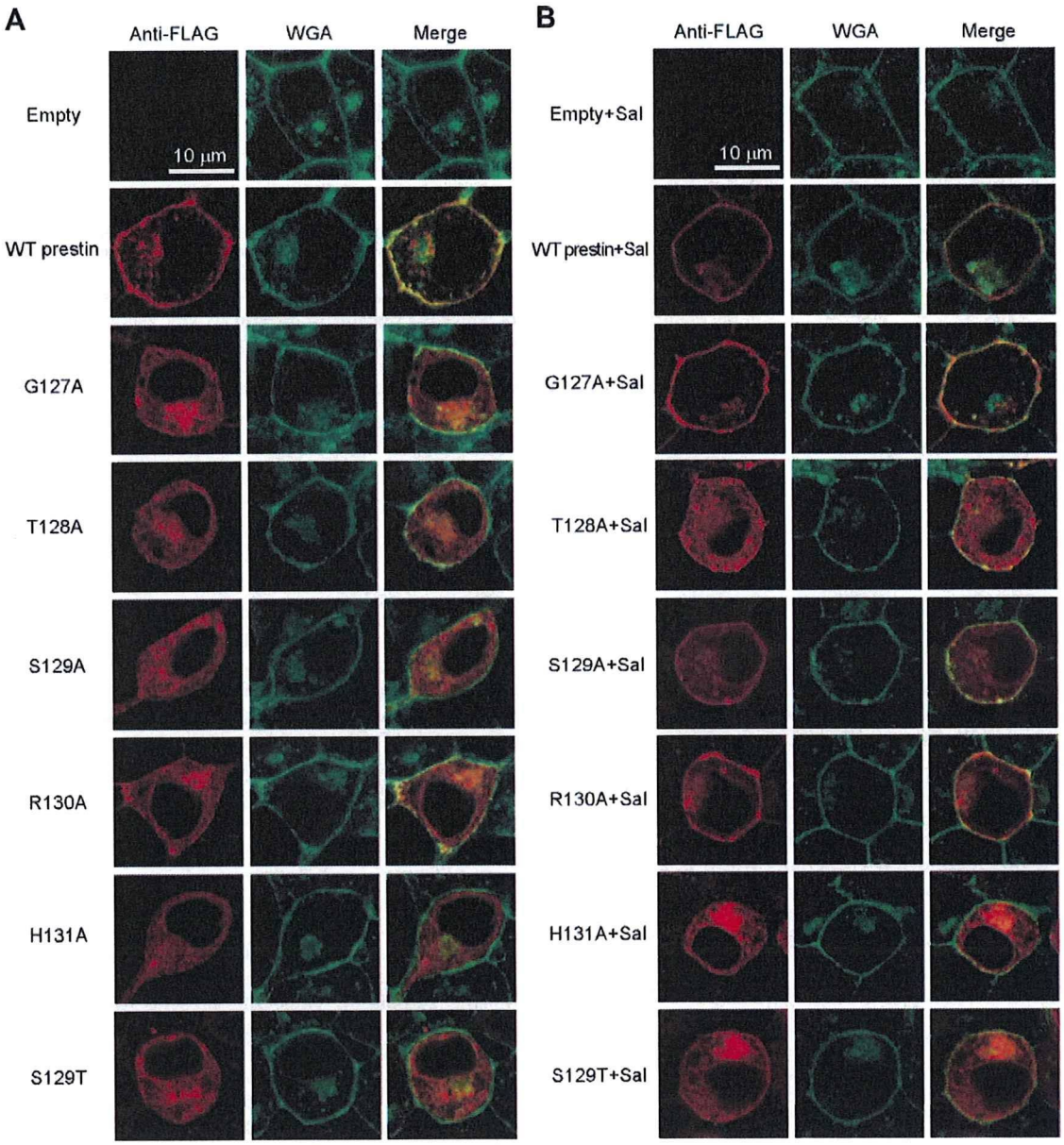


**Fig. 1.** Representative immunofluorescence images of transfected cells. (A) Stained cells cultured without 10 mM salicylate. (B) Stained cells cultured with 10 mM salicylate. Red and green fluorescence show prestin and both the plasma membrane and Golgi bodies, respectively. In the merged images, yellow–orange fluorescence indicates the co-localization of prestin and the plasma membrane, and that of prestin and the Golgi bodies.

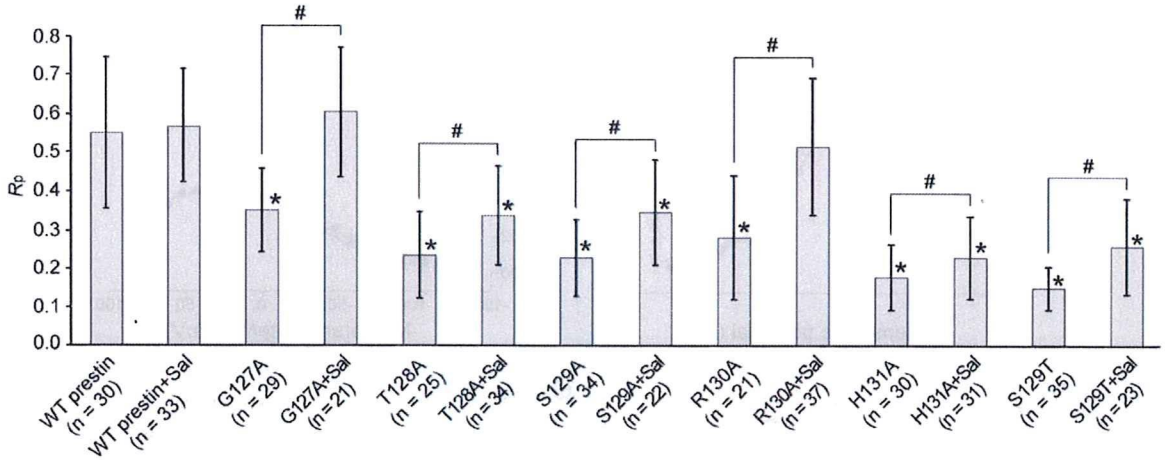


**Fig. 2.** $R_p$ of WT prestin and its mutants. The $R_p$ values of all prestin mutants were statistically lower than that of WT prestin when salicylate was not used, but they were increased by 10 mM salicylate. Asterisks show the statistical differences in the $R_p$ values between WT prestin and the prestin mutants and between WT prestin+Sal and the prestin mutants+Sal ($p < 0.05$). Number signs indicate statistical differences between $R_p$ values obtained from cells cultured with salicylate and those obtained from cells cultured without it in each prestin mutant ($p < 0.05$). Error bars show standard deviations.

each prestin mutant and the ratio of the amount of prestin in the plasma membrane to the total amount of prestin in the cell, $R_p$, was investigated. $R_p$ was calculated by the following equation:

$$R_p = \frac{I_p}{I_w}, \tag{1}$$

where $I_w$ is the sum of the intensity values of TRITC fluorescence of the whole area of the target cell which reflects the total amount of prestin in the cell, and $I_p$ is the sum of the intensity values of TRITC fluorescence of only the pixels corresponding to the plasma membrane, which reflects the amount of prestin there. The $I_w$ and $I_p$ were calculated as described in our previous study [12].

### 2.4. Evaluation of electrophysiological properties of prestin

NLC, which is generally used for the analysis of prestin activity, was measured in the whole-cell patch-clamp recording as described in our previous study [13]. Transfected cells were washed just before the recording. By such washing, salicylate bound to prestin in the plasma membrane was expected to be dissociated [9]. The cells without membrane disruption which showed robust GFP fluorescence were selected for measurement. The recorded membrane capacitance was fitted with the first derivative of the Boltzmann function [14],

$$C_m(V) = C_{lin} + \frac{Q_{max}}{\alpha e^{\frac{V-V_{1/2}}{\alpha}}\left(1 + e^{-\frac{V-V_{1/2}}{\alpha}}\right)^2}, \tag{2}$$

where $C_{lin}$ is the linear capacitance, which is proportional to the membrane area of the cells, $Q_{max}$ is the maximum charge transfer, $V$ is the membrane potential and $V_{1/2}$ is the voltage at half-maximal charge transfer. In Eq. (2), $\alpha$ is the slope factor of the voltage-dependent charge transfer and is given by

$$\alpha = kT/ze, \tag{3}$$

where $k$ is Boltzmann's constant, $T$ is absolute temperature, $z$ is valence and $e$ is electron charge. To evaluate the maximum charge transfer of prestin in the unit plasma membrane, $Q_{max}$, which means the maximum charge transfer of prestin in whole plasma membrane, was divided by $C_{lin}$ and designated as charge density.

For the comparison of NLC curve, NLC had to be normalized by the area of the plasma membrane. The normalized NLC $C_{nonlin/lin}$ was defined as

$$C_{nonlin/lin}(V) = \frac{C_{nonlin}}{C_{lin}} = \frac{(C_m(V) - C_{lin})}{C_{lin}}, \tag{4}$$

where $C_{nonlin}$ is the nonlinear component of the measured membrane capacitance.

### 2.5. Concentration dependence of effects of salicylate on prestin

The relationship between the concentration of salicylate and the degree of the promotion of the plasma membrane expression of prestin mutants was investigated. The cells transfected with R130A were cultured with sodium salicylate at concentrations of 1 mM and 5 mM from 12 h after transfection. By the above-mentioned method, after 24 h of incubation, the cells were subjected to immunofluorescence staining and the $R_p$ was then calculated.

## 3. Results and discussion

### 3.1. Localization of prestin in transfected cells

Representative immunofluorescence images of stained cells which were cultured without and with 10 mM salicylate are shown in Fig. 1A and B, respectively. To statistically investigate the localization of prestin in the cells, the $R_p$ was calculated and shown in Fig. 2. Without salicylate, the $R_P$ values of the prestin mutants were statistically lower than that of WT prestin ($p < 0.05$), suggesting that those mutants were accumulated in the cytoplasm. To confirm whether or not salicylate has the ability to promote the plasma membrane expression of the prestin mutants, prestin-transfected cells were cultured with 10 mM salicylate. The $R_p$ of WT prestin was unchanged by 10 mM salicylate, indicating that such amount of salicylate did not affect the process of transport of WT prestin to the plasma membrane (Fig. 2). On the other hand, the $R_P$ values of all prestin mutants statistically increased, compared with those when salicylate was not used ($p < 0.05$). Especially, the $R_p$ of G127A+Sal and that of R130A+Sal were similar to that of WT prestin+Sal. These results indicate that salicylate promoted the plasma membrane expression of the prestin mutants accumulated in the cytoplasm.

### 3.2. Electrophysiological properties of prestin

The $C_{nonlin/lin}$ (V), and charge density and $\alpha$ of WT prestin and its mutants are shown in Figs. 3 and 4, respectively. Without

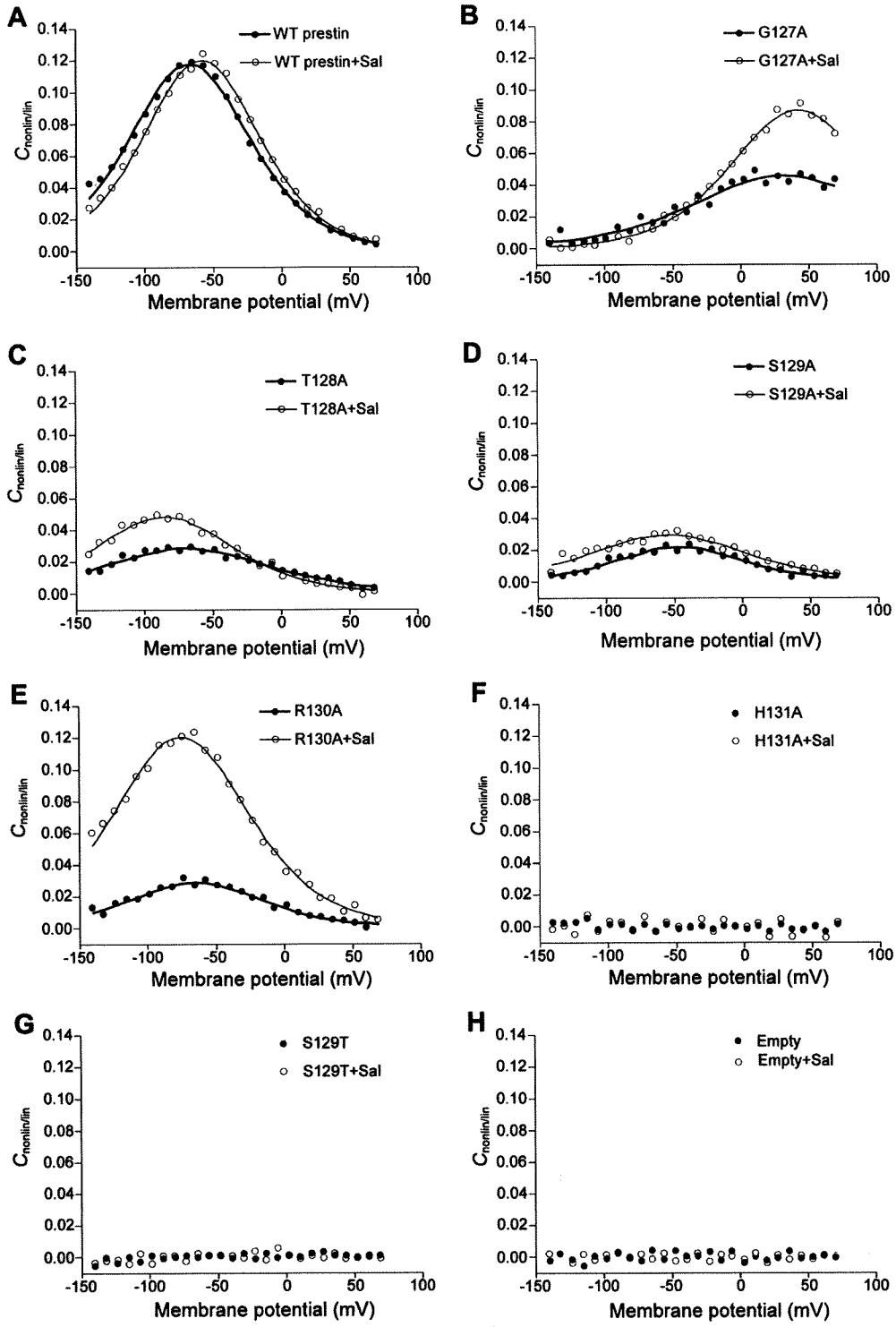


**Fig. 3.** Effects of salicylate on $C_{nonlin/lin}$ of prestin. Filled circles and thick lines show the results of transfected cells cultured without salicylate, while open circles and thin lines indicate the results of transfected cells cultured with 10 mM salicylate. (A) WT prestin and WT prestin+Sal. (B) G127A and G127A+Sal. (C) T128A and T128A+Sal. (D) S129A and S129A+Sal. (E) R130A and R130A+Sal. (F) H131A and H131A+Sal. (G) S129T and S129T+Sal. (H) Empty and Empty+Sal. When salicylate was used, NLC of G127A, T128A, S129A or R130A increased. On the other hand, in the case of H131A and S129T, NLC could not be detected in either type of cell, namely, cells cultured with and without 10 mM salicylate.

salicylate, G127A, T128A, S129A and R130A exhibited NLC, although their charge density was statistically smaller than that of WT prestin. On the other hand, H131A and S129T did not show NLC. The charge density of WT prestin+Sal was similar to that of WT prestin, suggesting that salicylate did not affect WT prestin itself nor the properties of the cells involved in the function of

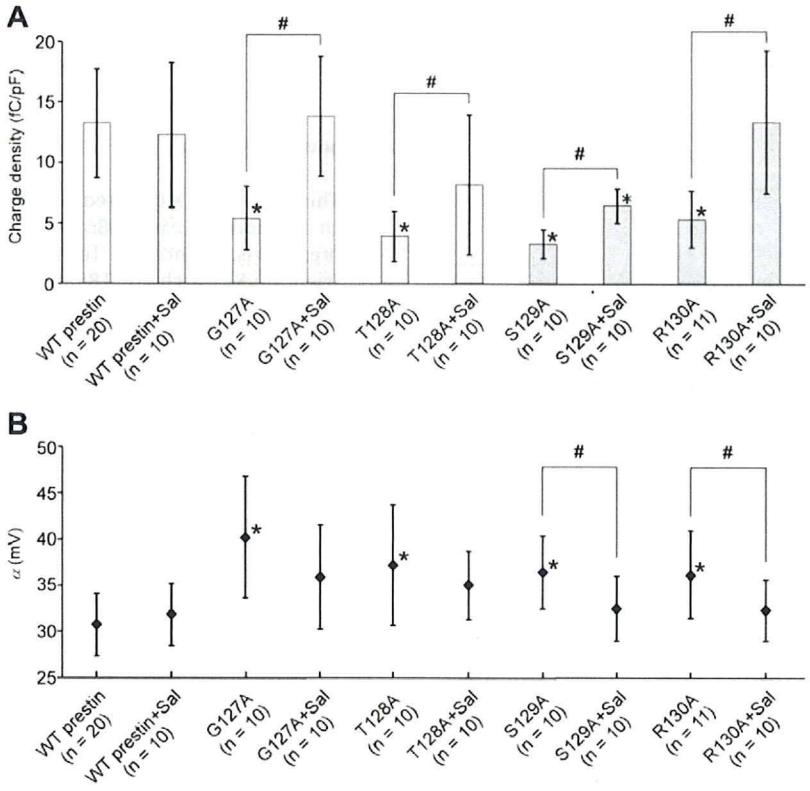


**Fig. 4.** Changes in the charge density and $\alpha$ of prestin by salicylate. (A) Charge density. The charge density of WT prestin was not affected by salicylate. On the other hand, the charge densities of G127A+Sal, T128A+Sal, S129A+Sal and R130A+Sal were statistically larger than those of G127A, T128A, S129A and R130A, respectively. (B) $\alpha$. Without salicylate, the $\alpha$ values of G127A, T128A, S129A and R130A were statistically different from that of WT prestin. On the other hand, when 10 mM salicylate was used, there was no statistical difference in the $\alpha$ between the prestin mutants and WT prestin. Asterisks show the statistical differences in the charge density and $\alpha$ between WT prestin and the prestin mutants and between WT prestin+Sal and the prestin mutants+Sal ($p < 0.05$). Number signs indicate statistical differences in the charge density and $\alpha$ between cells cultured with salicylate and those cultured without it in each prestin mutant ($p < 0.05$). Error bars represent standard deviations.

prestin. On the other hand, the charge densities of G127A+Sal, T128A+Sal, S129A+Sal and R130A+Sal were statistically larger than those of G127A, T128A, S129A and R130A, respectively ($p < 0.05$). Especially, the charge density of G127A+Sal and that of R130A+Sal were similar to that of WT prestin+Sal. These results indicate that the charge density of those four mutants was recovered due to salicylate. On the other hand, H131A and S129T did not show NLC even when transfected cells were cultured with 10 mM salicylate.

The $\alpha$ was considered to represent properties of the anion binding of prestin [15]. Such values of G127A, T128A, S129A and R130A were statistically different from that of WT prestin when salicylate was not used (Fig. 4). On the other hand, when transfected cells were cultured with 10 mM salicylate, there was no statistical difference in $\alpha$ between the prestin mutants and WT prestin (Fig. 4). These results may imply that culturing the cells with salicylate somehow affects the properties of the anion binding of prestin.

### 3.3. Correlation between the $R_p$ and the charge density

Without salicylate, the $R_p$ values of all prestin mutants were lower than that of WT prestin. In this condition, the charge density of the prestin mutants was also lower or not recorded. On the other hand, salicylate increased both $R_p$ and the charge density of G127A, T128A, S129A and R130A. Especially in G127A and R130A, $R_p$ as well as the charge density recovered to the WT prestin level. This trend suggests that the changes in the charge density were correlated with changes in the $R_p$. Although $R_p$ increased to some degree due to the addition of salicylate, H131A and S129T did not show NLC, possibly indicating that the amount of those mutants in the plasma membrane was still insufficient for detection of NLC. Another possibility is that H131A and S129T were promoted to be expressed in the plasma membrane but were non-functional.

Regarding S129A and S129T, the replacement of Ser-129 by threonine affected both the $R_p$ and the charge density of prestin more strongly than that by alanine. Alanine and threonine are, respectively, smaller and larger than serine. Thus, the existence of an amino acid larger than serine at position 129 of prestin may be a steric constraint, affecting its characteristics significantly.

### 3.4. Changes in the concentration of salicylate

Salicylate at the concentration of 10 mM was found to recover the plasma membrane expression and the charge density of G127A and R130A to the WT prestin level as described above. Effects of decreasing the concentration of salicylate from 10 mM to 5 mM and 1 mM on the promotion of the plasma membrane expression were then evaluated using the cells transfected with R130A. Confocal images of the stained cells and calculated $R_p$ are shown in Fig. 5A and B, respectively. The $R_p$ of R130A was unchanged by 1 mM salicylate, while it was increased by 5 mM salicylate but not to the WT prestin level, suggesting that the promotion by salicylate of the plasma membrane expression of prestin mutants was concentration-dependent.

### 3.5. Discovery of new effect of salicylate on prestin

Salicylate is generally known to be an antagonist of prestin [8,9]. In the present study, another feature of salicylate was

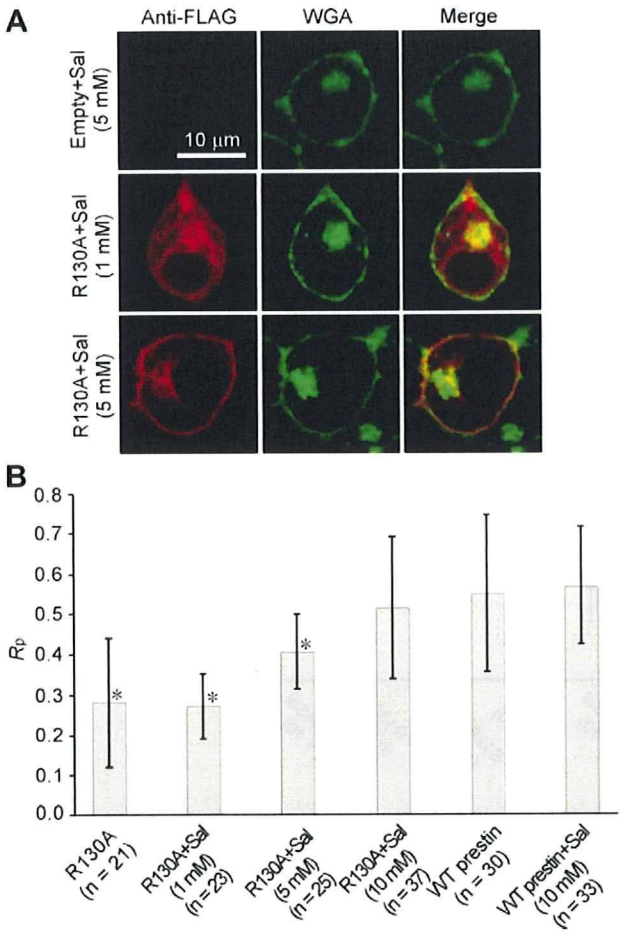


**Fig. 5.** Concentration dependence of the effects of salicylate on the localization of prestin. (A) Confocal microscopy images of stained cells. (B) Difference in the $R_p$ due to the difference in the concentration of salicylate. The samples of R130A-expressing cells cultured without salicylate, with 1 mM salicylate, with 5 mM salicylate and with 10 mM salicylate are termed R130A, R130A+Sal (1 mM), R130A+Sal (5 mM) and R130A+Sal (10 mM), respectively, in this figure. In addition, the samples of WT prestin-expressing cells cultured without salicylate and with 10 mM salicylate are termed WT prestin and WT prestin+Sal (10 mM), respectively. The $R_p$ of R130A was unchanged by 1 mM salicylate, but was increased by 5 mM salicylate. When transfected cells were cultured with 10 mM salicylate, the $R_p$ recovered to the WT prestin level. Asterisks represent significance vs. WT prestin+Sal (10 mM) ($p < 0.05$). Error bars indicate standard deviations.

discovered, namely, it can promote the plasma membrane expression of prestin mutants accumulated in the cytoplasm, resulting in the recovery of the charge density. Various research findings have reported that if membrane proteins were accumulated in the cytoplasm due to their misfolding, a pharmacological chaperone bound to these proteins and then promoted their correct folding, resulting in their plasma membrane expression [3–7]. These reports may lead to a speculation that the prestin mutants analyzed in the present study were misfolded in the cytoplasm and that salicylate bound to these mutants and then induced their correct folding, promoting their transport to the plasma membrane. The next step of our study is to clarify if such speculation is correct, namely, to investigate the mechanism underlying the salicylate-induced recovery of the plasma membrane expression of prestin mutants.

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# Atomic force microscopy imaging of the structure of the motor protein prestin reconstituted into an artificial lipid bilayer

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ABSTRACT

**Prestin is the motor protein of cochlear outer hair cells and is essential for mammalian hearing. The present study aimed to clarify the structure of prestin by atomic force microscopy (AFM). Prestin was purified from Chinese hamster ovary cells which had been modified to stably express prestin, and then reconstituted into an artificial lipid bilayer. Immunofluorescence staining with anti-prestin antibody showed that the cytoplasmic side of prestin was possibly face up in the reconstituted lipid bilayer. AFM observation indicated that the cytoplasmic surface of prestin was ring-like with a diameter of about 11 nm.**



## 1. Introduction

The basis of electromotility of outer hair cells (OHCs) which realizes the high sensitivity of mammalian hearing is considered to be the motor protein prestin [1]. Several characteristics of prestin have been gradually clarified [2]. Murakoshi et al. [3] detected prestin in the plasma membrane of prestin-transfected Chinese hamster ovary (CHO) cells using Qdots as topographic markers and observed ring-like structures, possibly prestin, by atomic force microscopy (AFM). Mio et al. [4] observed prestin purified from prestin-transfected insect cells by transmission electron microscopy (TEM) and found prestin to be a bullet-shaped molecule. Although those two studies are significant, their observed images differed, indicating that the structure of prestin was unclear. Thus, the aim of the present study was to clarify such structure by reconstitution of purified prestin into an artificial lipid bilayer and observation of the prestin-reconstituted lipid bilayer by AFM.

## 2. Materials and methods

### 2.1. Purification of prestin

The purification of prestin was performed by the method established in our previous study with some modifications [5]. CHO cells which had been modified to stably express C-terminal 3×FLAG-tagged prestin were suspended in Tris–KCl buffer (10 mM Tris, 150 mM KCl, pH 7.4) and sonicated, followed by centrifugation at 1000×*g* for 7 min at 4 °C to remove nuclei and undisrupted cells. The obtained supernatant was centrifuged at 20360×*g* at 4 °C for 2 h to collect the membrane fraction of the cells. Membrane proteins were solubilized by resuspending the obtained membrane fraction in Tris–KCl buffer containing 10 mM *n*-nonyl-β-D-thiomaltopyranoside (NTM, Dojindo, Kumamoto, Japan). After 3-h incubation on ice, samples were centrifuged at 20360×*g* at 4 °C for 3 h to remove non-solubilized proteins. The supernatant was applied to a column filled with anti-FLAG affinity gel (Sigma–Aldrich, St. Louis, MO). The column was then washed with Tris–KCl buffer containing 0.065 mM Fos-Cholin-16 (Anatrace, Maumee, OH) to replace the detergent NTM with Fos-Choline-16. Afterward, prestin was competitively eluted with 1 ml of that buffer containing 500 μg/ml of 3×FLAG peptide (Sigma–Aldrich). Whether prestin was purified or not was confirmed by SDS–PAGE, followed by Western blotting with anti-FLAG antibody and HRP-conjugated anti-mouse IgG antibody and by silver staining.

*Abbreviations:* OHC, outer hair cell; CHO, Chinese hamster ovary; AFM, atomic force microscopy; TEM, transmission electron microscopy

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### 2.2. Reconstitution of prestin into a preformed lipid bilayer

The method of direct reconstitution of membrane proteins into a preformed lipid bilayer was applied in the present study [6]. An artificial lipid bilayer was formed on mica using dioeoyl-phosphatidylcholine (DOPC) and dipalmitoyl-phosphatidylcholine (DPPC) (Avanti Polar Lipids, Alabaster, AL). The lipid bilayer was preincubated for 30 min at 4 °C with Tris–KCl buffer containing 5 mM $CaCl_2$ and 0.0065 mM Fos-cholin-16 for equilibration of the detergent within the lipid bilayer. Afterward, such bilayer was incubated with Tris–KCl buffer containing purified prestin, 5 mM $CaCl_2$ and 0.039 mM Fos-cholin-16 for 15 min at 4 °C. Excess prestin was then removed by extensive rinsing with Tris–KCl buffer. As a negative control, the lipid bilayer treated with detergent but without prestin was also prepared.

### 2.3. Staining of prestin in the reconstituted lipid bilayer

The existence of prestin in the lipid bilayer was confirmed by immunofluorescence staining. The prestin-reconstituted lipid bilayer was incubated with Block Ace (Dainippon Pharmaceutical Co. Osaka, Japan) for 30 min at 37 °C to avoid non-specific binding of antibodies. Afterward, that bilayer was stained with goat anti-prestin N-terminus primary antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) at a dilution of 1:100 in PBS overnight at 4 °C and with anti-goat IgG Texas Red (Santa Cruz Biotechnology) at a dilution of 1:200 in PBS at 37 °C for 60 min. The stained lipid bilayer was observed by confocal microscopy.

### 2.4. AFM imaging

The height images of the lipid bilayer were acquired in Tris–KCl buffer filtered with a 0.2-μm nylon filter by Multimode V AFM with a Nanoscope V controller (Veeco, Santa Barbara, CA) at 24–26 °C. V-shaped $Si_3N_4$ cantilevers (OMCL-TR400PSA-2, Olympus, Tokyo, Japan) with a spring constant of 0.06 N/m were used. The AFM was operated in the oscillation imaging mode (Tapping mode™, Digital Instruments) at a scan frequency of 1–0.5 kHz. In the present study, three types of images were obtained by AFM, namely, low- (5.0 × 5.0 μm), middle- (1.0 × 1.0 μm) and high-magnification images (300 × 300 nm). Each scan line has 256 and 512 points of data and an image consists of 256 and 512 scan lines for low magnification images and for middle- and high-magnification images, respectively. Obtained AFM images were flattened by use of a software program (NanoScope v7.00, Veeco) to eliminate background slopes and to correct dispersions of individual scanning lines. In addition, only high-magnification images were low-pass filtered to reduce high frequency noise. When the observed structure was ring-like, the distance between two peaks based on the cross sections was taken to be its diameter, as was done in our previous study [3].

## 3. Results

### 3.1. Purification of prestin

Whether prestin was indeed purified or not was investigated by SDS–PAGE, followed by Western blotting and silver staining. Results of Western blotting and silver staining are shown in Fig. 1A and B, respectively. In the Western blotting image, the 100 kDa band, probably showing prestin, was detected. In the results of silver staining, only one band corresponding to the band observed in Western blotting was recognized.

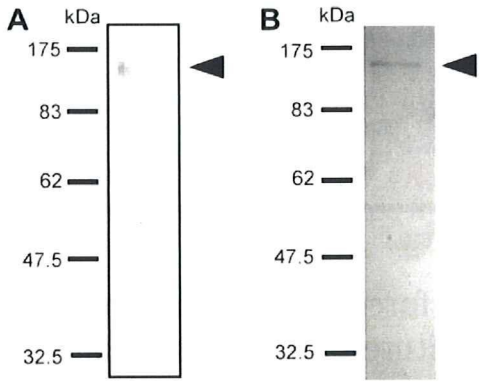


**Fig. 1.** Results of Western blotting and silver staining. (A) Western blotting data. A 100 kDa band probably showing prestin is seen. (B) Result of silver staining of SDS–PAGE gel. Only one band at 100 kDa, which was thought to correspond to the band detected in the result of Western blotting, is recognized.

### 3.2. Immunofluorescence staining of the prestin-reconstituted lipid bilayer

After the reconstitution process, immunofluorescence staining was employed to investigate whether prestin had been incorporated into the preformed lipid bilayer. Representative immunofluorescence images of the prestin-reconstituted lipid bilayer and negative control sample are shown in Fig. 2. Red fluorescence was detected in the prestin-reconstituted lipid bilayer but not in the negative control sample.

### 3.3. AFM imaging of the lipid bilayer

The AFM height image of the lipid bilayer without treatment showed two kinds of flat domains (Fig. 3A). A similar image was also obtained from the negative control sample (Fig. 3B). Unlike those two images, in addition to the flat domain, bumpy domains indicated by white arrows were detected in the low magnification AFM image of the prestin-reconstituted lipid bilayer (Fig. 3C). The boxed area in Fig. 3C was scanned by AFM and the obtained image is depicted in Fig. 3D. Dense small particles, some of which were recognized as ring-like structures, can be observed in that image. To clearly visualize the observed particles, the boxed area in Fig. 3D was scanned by AFM, the acquired image being shown in Fig. 3E. Moreover, three-dimensional representation of Fig. 3E is depicted in Fig. 4. Many ring-like structures were confirmed to be densely embedded in the lipid bilayer. The average diameter of such structures in Fig. 3E and other AFM images which are not shown here is 11.0 ± 1.3 nm ($n$ = 42).

## 4. Discussion

### 4.1. Reconstitution of prestin into an artificial lipid bilayer

After the purification process, only the 100 kDa band corresponding to the band in Western blotting data was detected by silver staining of SDS–PAGE gel, indicating that prestin had been purified. The 100 kDa band probably shows the monomer of prestin. As SDS possibly affects the binding between prestin molecules, to clearly confirm the oligomerization of purified prestin, a mild detergent such as perfluoro-octanoic acid should be used as in the study by Zheng et al. [7]. The AFM height image of the lipid bilayer without treatment shows two types of flat domains (Fig. 3A), as seen in previous studies [6,8–11]. At 24–26 °C, DOPC forms fluid-phase domains, while DPPC forms gel-phase domains. The thickness of DPPC in the gel-phase is larger than that of DOPC in

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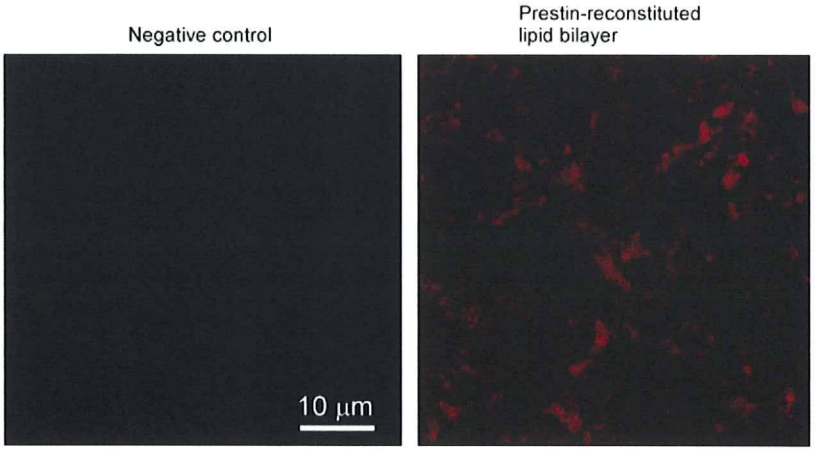


**Fig. 2.** Immunofluorescence staining of prestin-reconstituted lipid bilayer. Negative control sample and the prestin-reconstituted lipid bilayer were stained with anti-prestin antibody and anti-goat IgG Texas Red. Red fluorescence indicating the existence of prestin is only found in the prestin-reconstituted lipid bilayer.

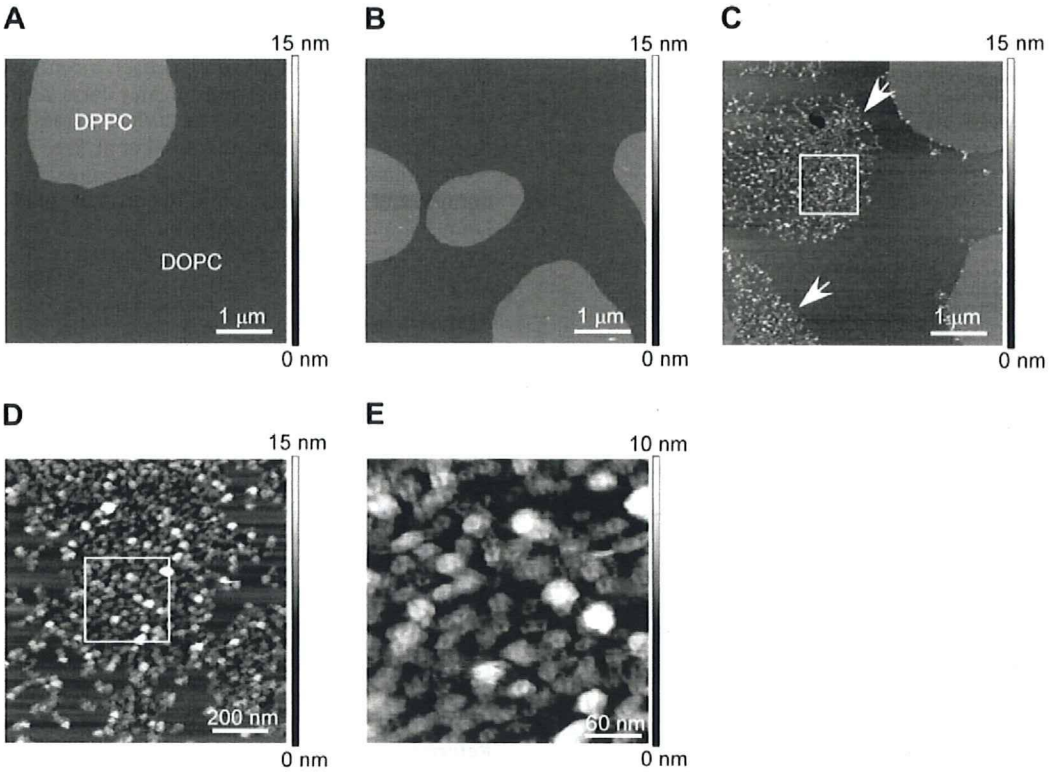


**Fig. 3.** AFM height images of the lipid bilayer. (A) AFM image of the lipid bilayer without treatment at low magnification. (B) AFM image of the negative control sample at low magnification. (C) AFM image of prestin-reconstituted lipid bilayer at low magnification. (D) Middle-magnification image obtained by scanning of the boxed area shown in (C). (E) High-magnification image obtained by scanning of the boxed area shown in (D). Two kinds of flat domains in (A) and (B) probably represent the domain of DPPC in the gel-phase and that of DOPC in the fluid-phase. Bumpy domains indicated by white arrows can be detected in the prestin-reconstituted lipid bilayer shown in (C). Many small particles can be found in the middle-magnification AFM images and those particles were recognized as ring-like in the high-magnification image. Such ring-like structures probably show prestin molecules.

the fluid-phase, thus indicating that the two types of observed domains were due to the difference in the thickness between the two lipids. After the reconstitution process, immunofluorescence staining using anti-prestin antibody showed that prestin existed in the reconstituted lipid bilayer (Fig. 2). In the AFM image, the bumpy domains, which probably corresponded to prestin, were recognized only in DOPC domains of the prestin-reconstituted lipid bilayer. Milhiet et al. [6] have also suggested that proteins of interest were reconstituted only into the DOPC domains in the fluid state. Thus, the present study and their study imply that proteins tend to be reconstituted into the DOPC domains in the fluid state. In the AFM image at high-magnification, ring-like structures probably showing prestin were densely reconstituted into the lipid bilayer. However, the alignment of such structures as found in the OHC plasma membrane by Sinha et al. [12] was not detected, which might have resulted from differences in the environment

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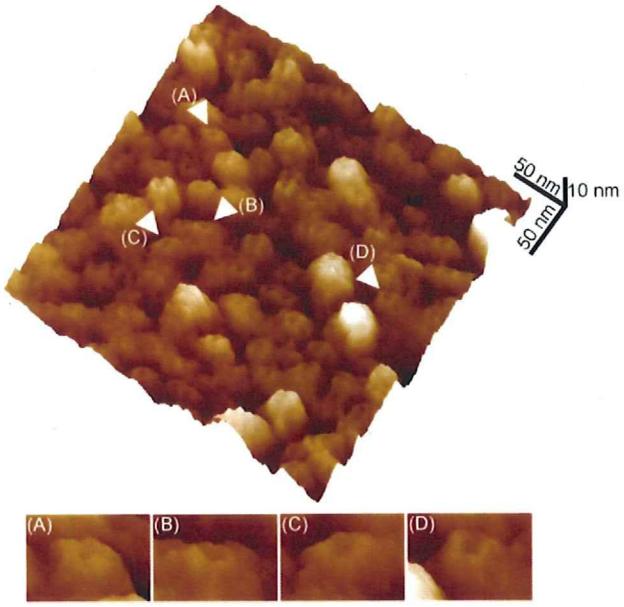


**Fig. 4.** Three-dimensional AFM height image of the prestin-reconstituted lipid bilayer. This figure was created from Fig. 3E. Representative examples of ring-like structures were digitally magnified and are shown (A and B). Ring-like structures, considered to be similar to those observed in the study of Murakoshi et al. [3], are found to be densely embedded in the lipid bilayer.

**Table 1**
Comparison of the size of prestin.

| Sample | Method | Diameter (nm) | References |
|---|---|---|---|
| OHC plasma membrane | AFM | 11–25 | Le Grimellec et al. [15] |
| Purified prestin | TEM | 7.7–9.6 | Mio et al. [4] |
| Prestin-expressing CHO cell plasma membrane | AFM | 9.6/13.0 | Murakoshi et al. [3] |
| OHC plasma membrane | AFM | 10 | Sinha et al. [12] |
| Prestin-reconstituted lipid bilayer | AFM | 11.0 ± 1.3 | This study |

between the OHC plasma membrane and the artificial lipid bilayer. The existence of actin cytoskeleton in OHCs and that of mica in the present study would affect the alignment of prestin.

### 4.2. Orientation of prestin

The orientation of prestin should be considered to confirm which side of prestin was observed by AFM, the extracellular side or the cytoplasmic side. Previous reports have suggested that when membrane proteins were reconstituted into a preformed lipid bilayer as done in the present study, their unidirectional orientation was obtained [6,13,14], indicating that all prestin molecules reconstituted into the lipid bilayer might be oriented in the same direction. In the present study, the standard deviation of the diameter of the observed ring-like structure, 1.3 nm, was small. Small standard deviation might increase the possibility that only either prestin molecules whose extracellular side was exposed or such molecules whose cytoplasmic side was exposed existed in the reconstituted lipid bilayer. Data showed in the previous reports, small standard deviation of the diameter and successful staining of such bilayer with anti-prestin antibody which binds to the cytoplasmic side of prestin possibly implied that the cytoplasmic side of prestin was face up. Although the possibility that the extracellular side of a few prestin molecules was exposed was not completely ruled out, it was considered that AFM possibly visualized the cytoplasmic side of prestin in the present study.

### 4.3. Structure and size of prestin

The AFM image of the prestin-reconstituted lipid bilayer showed dense ring-like structures, each with a diameter of 11.0 ± 1.3 nm, which were probably the surface structure of the cytoplasmic side of prestin. The previously reported sizes of prestin obtained by observation of the cytoplasmic side of prestin are listed in Table 1 [3,4,12,15]. Although it is unclear whether the particles detected in OHC plasma membranes are only comprised of prestin or not, our result is consistent with the previously reported sizes, supporting the assumption that the observed structures were prestin.

Le Grimellec et al. [15] found structures with a central depression in the cytoplasmic side of the OHC plasma membrane by AFM. Murakoshi et al. [3] showed by AFM that prestin might form a ring-like structure. On the other hand, Mio et al. [4] suggested that prestin is a bullet-shaped molecule which protrudes into the cytoplasmic side. Although Sinha et al. [12] found 10-nm particles in the cytoplasmic side of the OHC plasma membrane by AFM, whether those particles were ring-like or not was not specified. Thus, the structure of prestin has been a controversial issue. Our results demonstrate that prestin may form a ring-like structure with a diameter of about 11 nm, which agrees with results of Le Grimellec et al. [15] and Murakoshi et al. [3].

In summary, the present study attempted to visualize prestin purified and reconstituted into the artificial lipid bilayer by AFM. From the obtained AFM image, the cytoplasmic surface of prestin was indicated to be ring-like with a diameter of about 11 nm.

## Acknowledgements

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Pediatrics of Japan
小 児 科

特集：*小児疾患における臨床遺伝学の進歩*

# 先天性難聴

宇佐美 真 一

金 原 出 版 株 式 会 社

### 7．皮膚脆弱型EDS（dermatosparaxis type MIM225410）

皮膚脆弱型EDSは顕著で皮膚過伸展性は強く，出血しやすい．創傷治癒に問題なく，瘢痕形成はない．他の徴候として泉門の閉鎖が遅れたり，特徴的顔貌，眼瞼浮腫，青色強膜，臍ヘルニア，短指や低身長がみられる．皮膚脆弱型EDSはⅠ型プロコラーゲンN-ペプチダーゼ自体の遺伝子異常による常染色体劣性遺伝病である．

## Ⅱ 診断・鑑別診断

臨床症状と家族歴や類似疾患との鑑別からどの病型のEDSであるかを明確にする．原因遺伝子が判明している場合，確定診断のために培養皮膚線維芽細胞による蛋白解析や遺伝子変異解析も可能である．常染色体優性遺伝形式をきたす病型は家族歴，家系図からも遺伝形式を判定できる．近年，6病型に分類できないEDSも報告されている[7]．

## Ⅲ 経過・予後・治療

血管型EDSを除き，一般的に生命予後は良好だが，病型，合併症の有無・程度により異なる．EDSは現在のところ全般的に根本的な治療はなく，対症療法が中心となる．多岐にわたる症状に対応するため，病型によっては他科との連携，とくに古典型では外科・形成外科，関節可動性亢進型では整形外科，血管型では緊急時の対応体制が求められる．リスクの高い活動を避ける生活や患児を取り巻く周辺環境に対する指導もとりいれた継続したフォローアップや，時に心理的サポートも求められる．

EDSは小児慢性特定疾患に含まれている．サポートグループとして，米国ではThe Ehlers-Danlos National Foundation（http://www.ednf.org），日本においても日本エーラスダンロス症候群協会（友の会）（http://ehlers-danlos-jp.net/modules/EDS2/）がある．

## おわりに

EDSは，結合組織に異常をきたす症候群であるが，病型により症状が異なり異質性が強い．しかしながら，時にある一病型の症状（例えば皮膚症状や血管症状）が本疾患を代表する印象をもたれ，多岐にわたる患児それぞれの本来の症状・状況に合わずに誤解して対応され，患児や家族にとって日常生活上支障をきたしている場合も見受けられる．本症候群においては，病型の周知と医療状況や環境の包括的な調整・支援が患児や両親にとって必要とされ，コーディネーターとしての小児科医の役割は重要であり期待されている．

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