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# Plant regeneration from cell suspension-derived protoplasts of *Saintpaulia ionantha* Wendl

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**Summary.** Friable calli were induced on leaf segments of *Saintpaulia ionantha* Wendl. on B5 medium containing 1 mg $l^{-1}$ 2,4-D and 2 g $l^{-1}$ casein hydrolysate. Cell suspension cultures were readily established from these friable calli and protoplasts could be isolated from the cells with yields of 1-3 x $10^7$/g f. wt.. By culturing in 0.1 % gellan gum-solidified B5 medium supplemented with 1 mg $l^{-1}$ 2,4-D and 0.1 M each of sucrose and mannitol at a density of 1 x $10^5$/ml, the protoplasts divided within 6 days and formed macro-colonies after 2 months of culture. Shoot regeneration from protoplast-derived calli was obtained by sequential treatment of the calli with plant growth regulators: initially with 1 mg $l^{-1}$ each of NAA and BA for 2 months followed by 0.01 mg $l^{-1}$ NAA and 5 mg $l^{-1}$ BA for 4 months. Regenerated plants were established after rooting of the shoots on half-strength MS medium, and successfully transferred to the greenhouse. The regenerated plants grew into flowering stage and showed the same phenotype as the parent plant.

*Abbreviations.* BA, benzyladenine; 2,4-D, 2,4-dichlorophenoxyacetic acid; FDA, fluorescein diacetate; f. wt., fresh weight; MES, 2-(N-morpholino)-ethanesulfonic acid; MS, Murashige and Skoog (1962); NAA, α-naphthaleneacetic acid; PE, plating efficiency.

## Introduction

The Gesneriaceae contains many important ornamental genera such as *Saintpaulia*, *Sinningia*, *Achimenes* and *Streptocarpus*. Among them, *Saintpaulia ionantha* Wendl., commonly called saintpaulia or African violet, involves numerous cultivars with varied flower color, leaf color and shape, and is one of the commercially most popular ornamental species. Breeding of this species has only been achieved by intraspecific hybridization and sport selection, and neither interspecific nor intergeneric hybridization has been incorporated (Grout 1990).

Recent developments of biotechnology such as somatic hybridization and genetic transformation provide additional means for further improvement of saintpaulia with respect to floral and marketable qualities. For utilizing these techniques, it is indispensable to establish an efficient system for protoplast culture to plant regeneration in this species. To date, several micropropagation systems have been developed for saintpaulia (Geier 1983; Smith and Norris 1983; Cassells and Plunkett 1984; Mølgaard *et al.* 1991). However, plant regeneration from saintpaulia protoplasts has not been demonstrated, although several reports on protoplast isolation and culture have appeared (Hughes 1977; Bilkey and Cocking 1982). In the present study, we describe successful regeneration of plants from cell suspension culture-derived protoplasts of saintpaulia.

## Materials and methods

*Plant materials.* Expanded young leaves, 4.0 to 5.0 cm long, of *Saintpaulia ionantha* Wendl. cv. Pink Veil purchased from local market were harvested from stock plants grown in a greenhouse and surface-sterilized with 1 % sodium hypochlorite solution for 10 min followed by 3 rinses with sterilized distilled water. Leaf laminae were cut into 5 mm square pieces and placed with the abaxial side down on MS medium (Murashige and Skoog 1962) supplemented with 1 mg $l^{-1}$ each of NAA and BA, 2 % (w/v) sucrose and 0.2 % (w/v) gellan gum (Gelrite; Kelco, Division of Merck and Co. Inc., San Diego, CA) in 90 x 20 mm plastic Petri dishes containing 40 ml of the medium. The pH of the culture media used for adventitious shoot induction from leaves and plantlet subculture was adjusted to 5.8 prior to autoclaving at 121 °C for 15 min. After 2 months of culture, adventitious shoots that developed from the leaf explants were excised and transferred to half-strength MS medium containing 2 % (w/v) sucrose and 0.2 % (w/v) gellan gum for rooting. Plantlets thus obtained were subcultured monthly on the same medium. Stock cultures were kept at 25 °C under 24 h illumination (35 $\mu$mol $m^{-2}$ $s^{-1}$) with fluorescent lamps (National FL30SN).

*Cell suspension cultures.* Leaf laminae, taken from *in vitro*-grown plantlets 3 weeks after subculture, were cut into 5 x 5 mm pieces and placed on 0.2 % (w/v) gellan gum-solidified B5 medium (Gamborg *et al.* 1968) supplemented with 2 % (w/v) sucrose and 0, 1, 5 or 10 mg $l^{-1}$ of auxins (NAA or 2,4-D) in combination with 0, 1, 5 or 10 mg $l^{-1}$ BA, with or without 2 g $l^{-1}$ casein hydrolysate (NZ Amine, Type A; Wako Pure Chemical Industries, Ltd.). For culture, 15 explants were placed in 90 x 20 mm plastic Petri dishes containing 40 ml of the medium. Two replicates of dishes were made for each treatment. The pH of the culture media was adjusted to 5.8 and the dishes were maintained at 25 °C in the dark.

For establishing cell suspension cultures, proliferating friable calli (ca. 1 g f. wt.) on the initial explants were transferred to 100 ml Erlenmeyer flasks, each of which contained 40 ml of B5 liquid medium supplemented

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with 1 mg $l^{-1}$ 2,4-D and 2 % (w/v) sucrose, at pH 5.8. Cell suspension cultures were maintained at 25 °C under 24 h illumination (35 $\mu$mol $m^{-2}s^{-1}$) on a gyratory shaker at 100 cycles $min^{-1}$. Subcultures were performed every 10 days by transferring ca. 0.8 g f. wt. of cells to 40 ml of fresh medium/flask.

*Protoplast isolation and culture.* Protoplasts were isolated from cell suspension cultures 4 days after subculture. One g f. wt. of suspension cells were incubated in 10 ml of filter-sterilized (Millipore, 0.45 $\mu$m pore size) enzyme solution containing 2 % (w/v) Cellulase Onozuka RS and 1 % (w/v) Macerozyme R-10 (both Yakult Pharmaceutical Co. Ltd., Japan), 0.05 % (w/v) Pectolyase Y-23 (Seishin Pharmaceutical Co. Ltd., Japan), 1 % (w/v) Driselase (Kyowa Hakko Kogyo Co. Ltd., Japan), 5 mM $CaCl_2 \cdot 2H_2O$, 5 mM MES and 0.2 M mannitol. The pH of the enzyme solution was adjusted to 5.8. After 4 h of incubation at 25 °C with gentle gyratory shaking (30 cycles $min^{-1}$), the mixture was passed successively through nylon sieves (60 and 30 $\mu$m) and the protoplasts were collected by centrifugation (120 x g, 3 min). Protoplasts were washed twice with half-strength MS medium containing 0.2 M mannitol by resuspension and centrifugation (120 x g, 3 min). Viability of protoplasts was assessed with FDA staining (Widholm 1972).

Protoplasts were cultured at 1 x $10^5$/ml in 35 x 10 mm plastic Petri dishes containing 2 ml of 0.1 % (w/v) gellan gum-solidified B5 medium. Twice the concentration of protoplasts, 2 x $10^5$/ml, in double-strength liquid medium was mixed with an equal volume of 0.2 % (w/v) gellan gum solution at 40 °C just before culture (Mii *et al.* 1991). Various combinations of auxins (NAA, 2,4-D or picloram), and sugars (sucrose, glucose or fructose) or sugar alcohol (mannitol) were evaluated in the culture medium. The pH of culture media was adjusted to 5.8. All dishes were sealed with Parafilm® and maintained at 25 °C in the dark. Plating efficiency (PE), defined as the percentage of dividing protoplasts, was obtained after 14 days of culture.

*Plant regeneration.* After 2 months of culture, protoplast-derived macro-colonies, ca. 1 mm diameter, were transferred to B5 medium containing 1 mg $l^{-1}$ 2,4-D in order to proliferate callus for a month. Protoplast-derived calli were then transferred onto B5 medium containing 1 mg $l^{-1}$ each of NAA and BA for callus preconditioning. After 2 months, these preconditioned calli were then transferred for shoot induction onto B5 media containing various concentrations of NAA (0, 0.01, 0.1 or 1 mg $l^{-1}$) and BA (0, 1 or 5 mg $l^{-1}$).

Regenerated shoots were detached from the callus and transferred for rooting to half-strength MS medium lacking plant growth regulators. All media used during and after callus proliferation were supplemented with 2 % sucrose and solidified with 0.2 % gellan gum, adjusted to pH 5.8. Cultures were maintained at 25 °C under 24 h illumination (35 $\mu$mol $m^{-2}$ $s^{-1}$) with fluorescent lamps (National FL30SN).

Regenerated plantlets with a well-established root system were washed carefully to remove the gellan gum and transferred to pots (9 x 9 cm) containing vermiculite. Potted plants were acclimatized in a transparent plastic cabinet covered with polyethylene bags at 20 °C under 24 h illumination (45 $\mu$mol $m^{-2}$ $s^{-1}$) with fluorescent lamps. After 2 to 3 weeks, acclimatized plants were transferred to the greenhouse.

## Results and Discussion

Protoplasts have previously been isolated mechanically or enzymatically from leaves, petioles or cultured petiole cross-sections of *in vitro*-grown saintpaulia plantlets (Hughes 1977; Bilkey and Cocking 1982). However, protoplast division leading to plant regeneration was not demonstrated in these studies. In our preliminary experiments, only a few protoplasts could be isolated from leaves of stock cultures by the enzyme solution and incubation environment used in this study (data not shown). On the other hand, it has been reported that cell suspension cultures were suitable as a donor source for protoplasts of several species including *Limonium perezii*, in which protoplast isolation from leaves was likewise very difficult or impossible (Kunitake and Mii 1990). Therefore, we initially attempted to induce friable calli from leaf disks of saintpaulia on B5-based medium containing 2,4-D or NAA in combination with BA, with or without casein hydrolysate (Table 1). On media containing NAA with or without casein hydrolysate, compact calli and/or adventitious shoots were preferentially produced, respectively. The compact calli produced on the media containing casein hydrolysate only proliferated as compact calli during the subculture on the same media. Friable calli were only induced on medium containing 1 mg $l^{-1}$ 2,4-D and 2 g $l^{-1}$ casein hydrolysate (Table 1), on which 70 % of leaf disks produced friable calli. However, these friable calli also proliferated when transferred to B5 medium without casein hydrolysate. These results indicate that casein hydrolysate may play an important role in induction of friable calli, but not be necessary for maintaining the calli obtained in this study. Casein hydrolysate has already been demonstrated to promote cell division, proliferation or plant regeneration in several tissue culture systems probably by providing amino acids (Ochatt and Power 1988; Kunitake and Mii 1990).

By transferring the leaf-derived friable calli into liquid B5 medium containing 1 mg $l^{-1}$ 2,4-D, fine cell suspension cultures were readily established, from which about a 5-fold increase in f. wt. was obtained within 10 days after subculture. Protoplasts were readily isolated from suspension cells 4 days after subculture and the successive size filtrations (Fig. 1A). Protoplast yields of 1-3 x $10^7$/g f. wt. suspension cells were routinely obtained and their viability was more than 90% as assessed with FDA.

Protoplasts started to divide after 3 to 6 days in culture (Fig 1B). The highest PE (12.7 %) was obtained in B5 medium with 0.2 M sucrose and 2,4-D (Table 2). The other two auxins also induced cell division, but with frequencies below 6 %.

**Table 1.** Effect of plant growth regulators and casein hydrolysate on adventitious shoot, root, friable callus and compact callus formation from saintpaulia leaf disks.

| | Casein hydrolysate (g $l^{-1}$) | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 0 | | | | | | 2 | | | | | |
| Plant growth regulators | NAA | | | | 2,4-D | | | NAA | | | 2,4-D | |
| (mg$l^{-1}$) | 0 | 1 | 5 | 10 | 1 | 5 | 10 | 0 | 1 | 5 | 10 | 1 | 5 | 10 | 1 | 5 | 10 |
| BA 0 | S,R | S,R | R | R | C | – | – | S,R | – | C | C | F | – | – |
| 1 | S | S | S,R | – | – | – | – | – | – | – | C | C | C | C |
| 5 | S | S | S | – | C | – | – | C | S | S,R | C | C | C | C |
| 10 | – | – | – | C | – | – | – | – | – | C | C | C | C | C |

All responses scored 2 months after culture.
S, adventitious shoot formation; R, adventitious root formation; F, friable callus formation; C, compact callus formation; –, no response.

**Table 2.** Effect of auxins on plating efficiency of saintpaulia protoplasts.

| Auxin (1 mg $l^{-1}$) | Plating efficiency (%) |
|---|---|
| NAA | 6.0 ± 1.3 |
| 2,4-D | 12.7 ± 1.4 |
| Picloram | 4.6 ± 0.5 |

Each value represents the mean ± SE of at least 3 independent experiments.

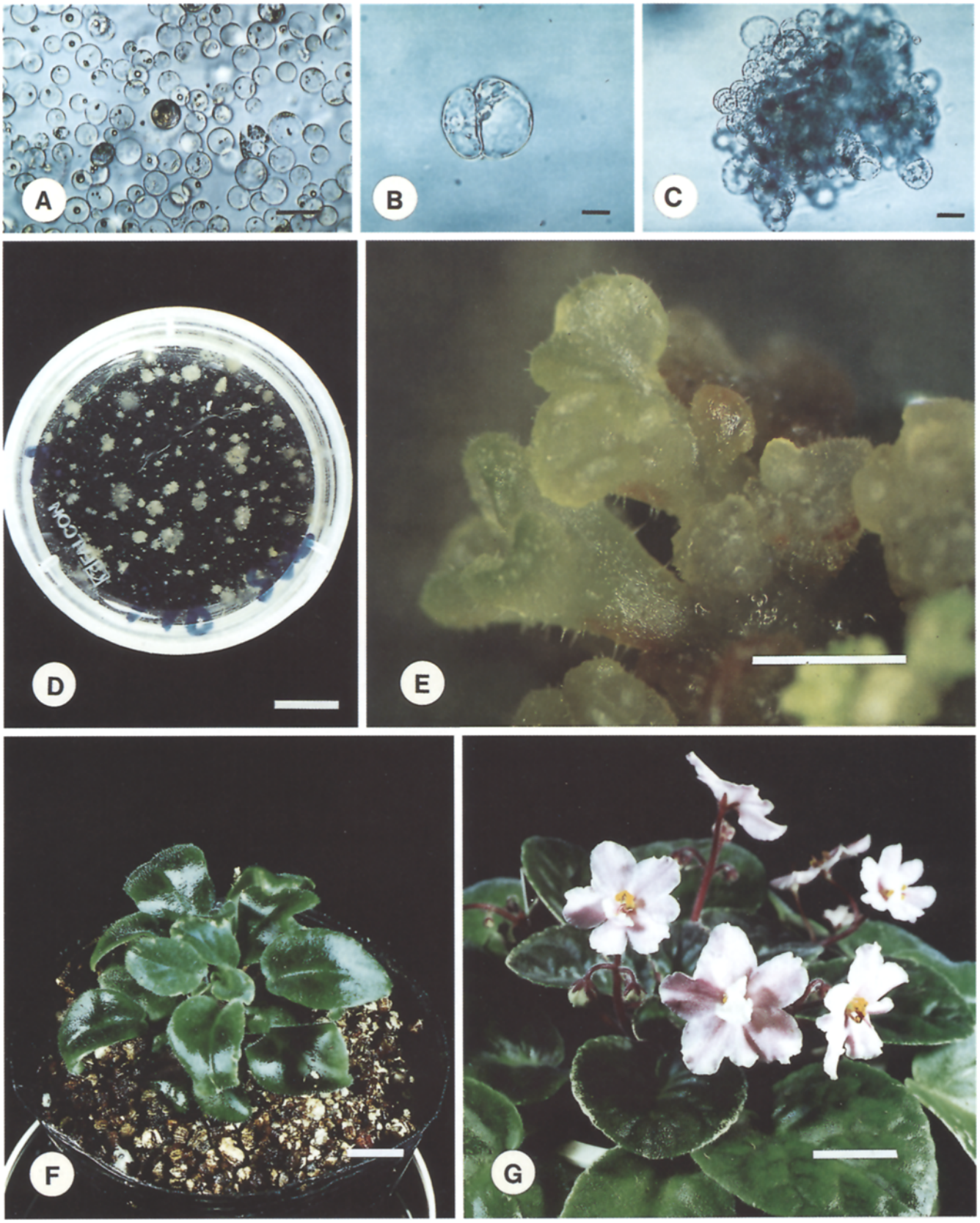


**Figure 1.** Plant regeneration from protoplasts of saintpaulia. (A) Protoplasts isolated from suspension cultured-cells. Bar = 40 $\mu$m. (B) First division of protoplasts after 5 days of culture. Bar = 15 $\mu$m. (C) Protoplast-derived colony after 2 months of culture. Bar = 90 $\mu$m. (D) Protoplast-derived visible colonies after 2 months of culture. Bar = 0.5 cm. (E) Adventitious shoot regeneration from a protoplast-derived callus on B5 medium containing 0.01 mg $l^{-1}$ NAA and 5 mg $l^{-1}$ BA 9 months after the initiation of protoplast culture. Bar = 1 cm. (F) A protoplast-derived plant established in a pot. Bar = 1 cm. (G) A regenerated plant in the flowering stage. Bar = 2 cm.

**Table 3.** Effect of sucrose concentration on plating efficiency of saintpaulia protoplasts.

| Sucrose (M) | Plating efficiency (%) |
|---|---|
| 0.1 | 0 |
| 0.2 | 12.9 ± 1.2 |
| 0.3 | 8.9 ± 0.8 |
| 0.4 | 7.3 ± 1.2 |
| 0.5 | 6.0 ± 1.0 |

Each value represents the mean ± SE of at least 3 independent experiments.

**Table 4.** Effect of sugars and sugar alcohol on plating efficiency of saintpaulia protoplasts.

| Carborn source | Plating efficiency (%) |
|---|---|
| 0.2M fructose | 0 |
| 0.2M glucose | 0 |
| 0.2M sucrose | 13.0 ± 1.1 |
| 0.1M sucrose + 0.1M mannitol | 18.6 ± 0.8 |
| 0.1M sucrose + 0.1M glucose | 4.5 ± 2.7 |
| 0.1M glucose + 0.1M mannitol | 0 |

Each value represents the mean ± SE of at least 3 independent experiments.

In B5 medium containing 0.1 M sucrose, protoplast bursting was frequently observed immediately after the cultures were initiated and no cell division occurred. Protoplasts most efficiently divided in B5 medium containing 0.2 M sucrose,12.9 %, (Table 3). Division frequency decreased as sucrose concentration increased over 0.2 M. Therefore, various sugars, sucrose, glucose or fructose, and sugar alcohol (mannitol) were also tested at 0.2 M singly or in combination (Table 4). Protoplast division was observed only in B5 media containing sucrose. The highest percentage of dividing protoplasts (18.6 %) was obtained from the medium containing 0.1 M sucrose in combination with 0.1 M mannitol. The other sugars, glucose and fructose, appeared inhibitory to cell division.

In several plant species, growth of protoplast-derived colonies has shown to be promoted by lowering the osmoticum concentration of the culture medium (Berry *et al.* 1982; Kunitake and Mii 1990). In saintpaulia, however, protoplasts cultured in gellan gum-solidified B5 medium containing 1 mg $l^{-1}$ of 2,4-D and 0.1 M each of sucrose and mannitol continued to divide and more than 200 macro-colonies per dish were formed after 2 months of culture without adding or changing the culture medium (Fig. 1C,D).

Macro-colonies developed into vigorously growing friable calli one month after transfer to the callus proliferation medium. These friable calli were transferred for preconditioning onto B5 medium containing 0.1 mg $l^{-1}$ each of NAA and BA, on which they became compact and occasionally developed adventitious roots after 2 months. Four months after transfer to shoot induction media, green shoot primordia appeared on the surface of the calli and adventitious shoots arose (Fig. 1E). Among the media tested, shoot regeneration occurred only on B5 medium supplemented with 0.01 mg $l^{-1}$ NAA and 5 mg $l^{-1}$ BA at a frequency of 27 %. On all other media, the calli continued to proliferate or eventually turned brown after 8 months of culture.

In this study, the preconditioning of protoplast-derived calli on B5 medium containing both NAA and BA seem to be essential for subsequent shoot induction. Neither calli without the preconditioning treatment nor those preconditioned with both 2,4-D and BA or with only NAA regenerated shoots. The importance of a preconditioning treatment prior to shoot regeneration has also been demonstrated for cultured cell-derived protoplasts of *Actinidia chinensis* (Mii and Ohashi 1988).

More than 100 shoots have so far regenerated from protoplast-derived calli of saintpaulia. Protoplast-derived shoots with 2 to 3 leaves were detached from the callus and transferred to half-strength MS medium, on which they readily developed roots. Regenerated plantlets were successfully established in the greenhouse 9 to 10 months after the initiation of protoplast cultures (Fig. 1F). Acclimatized plants exhibited a normal phenotype with respect to leaf shape and color, and flowered with normal color and morphology 6 months after transfer to the greenhouse (Fig. 1G). Production of both somatic hybrid plants and PEG- or electroporation-mediated transgenic saintpaulia plants using the protoplast culture system developed in this study are now in progress.

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