Oral Immunization with a Recombinant Bacterial Antigen Produced in Transgenic Plants

Tariq A. Haq, Hugh S. Mason, John D. Clements, Charles J. Arntzen*

The binding subunit of *Escherichia coli* heat-labile enterotoxin (LT-B) is a highly active oral immunogen. Transgenic tobacco and potato plants were made with the use of genes encoding LT-B or an LT-B fusion protein with a microsomal retention sequence. The plants expressed the foreign peptides, both of which formed oligomers that bound the natural ligand. Mice immunized by gavage produced serum and gut mucosal anti–LT-B immunoglobulins that neutralized the enterotoxin in cell protection assays. Feeding mice fresh transgenic potato tubers also caused oral immunization.

Vaccines against cholera and other enteric diseases would be a major benefit to developing countries where these diseases are often life threatening, especially in children. Efficacious vaccines must stimulate the mucosal immune system, leading to the production of secretory immunoglobulin A (IgA), a process better achieved by oral than by parenteral antigen delivery. Several particulate antigens, including both live and killed microorganisms, are effective oral immunogens (*1*), whereas subunit or soluble antigens are often relatively inefficient (*2*).

Enterotoxigenic *Escherichia coli* (ETEC) and *Vibrio cholerae* cause acute watery diarrhea by colonizing the small intestine and producing one or more enterotoxins, including the heat-labile enterotoxin (LT) of ETEC (*3*). The structure of this multimeric cholera toxin–like protein was recently elucidated (*4*). It has one 27-kD A subunit and a pentamer of 11.6-kD B (binding) subunits (LT-B). These are noncovalently linked into a very stable doughnut-like structure that binds to the G_{M1} ganglioside that is present on the epithelial cell surface. Antibody interference with binding of the B subunit to cells, thus blocking toxin activity, is the basis of attempts to use the B subunit as a vaccine component (*5*).

Two expression vectors (pLTB-110 and pLTK-110) were constructed (*6*) with the use of the gene encoding LT-B (Fig. 1A). *Agrobacterium tumefaciens* was used to transfer the constructs into tobacco (*Nicotiana tabacum* cv. Samsun) and potato plants (*Solanum tuberosum*, variety "Frito-Lay 1607") (*7*). Shoots were regenerated, transplanted to soil, and grown in lighted growth chambers. Antigen amounts in tobacco leaves and potato microtubers (*8*) were quantified by enzyme-linked immunosorbent assay (ELISA) based on ganglioside binding by LT-B (*9*) (Fig. 1, B and C). Because *Agrobacterium*-mediated transformation of plant cells results in random nuclear insertion of the transferred DNA (T-DNA) (*10*), individual transformants showed varying amounts of gene expression. Maximum amounts of protein accumulation in pLTB-110 plants were up to 5 μg per gram of total soluble leaf protein (Fig. 1B) and up to 30 μg per gram of soluble protein in potato microtubers (Fig. 1C). Extracts from pLTK-110 plants showed significantly elevated accumulation in comparison with extracts from pLTB-110 plants (Fig. 1, B and C). This suggests that the microsomal retention signal SEKDEL (*11*) caused cellular compartmentation of the fusion protein, thus facilitating oligomerization (*12*). The rLT-B–SEKDEL accumulated in amounts up to 14 μg per gram of total soluble tobacco leaf protein and 110 μg per gram of soluble potato micro-

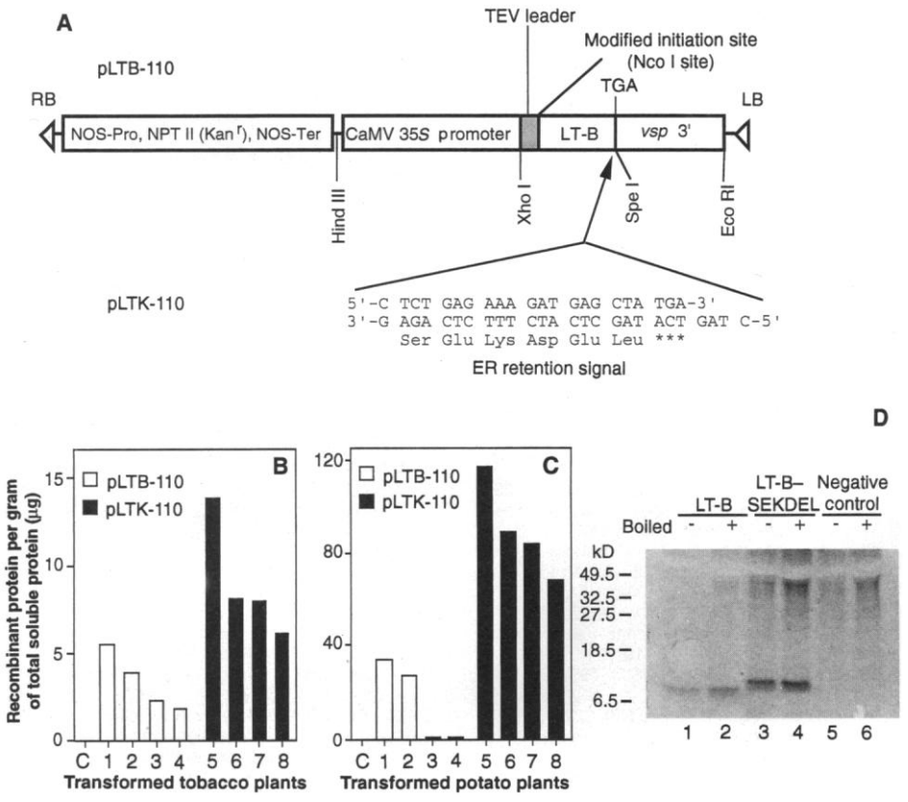

Fig. 1. Expression of LT-B and LT-B–SEKDEL in tobacco and potato plants. (**A**) The LT-B plant transformation vectors pLTB-110 and pLTK-110 (*6*) comprised an NPT II expression cassette for kanamycin resistance and an LT-B expression cassette with CaMV 35*S* promoter, TEV 5′-UTR, and *vspB* 3′ flank. In pLTK-110, DNA encoding the polypeptide SEKDEL was ligated 3′ of the gene encoding LT-B for endoplasmic reticulum (ER) retention of the protein. (**B**) Amounts of recombinant LT-B (pLTB-110) and LT-B–SEKDEL (pLTK-110) in soluble protein extracts from leaves of independent tobacco transformants. (**C**) Amounts of recombinant LT-B (pLTB-110) and LT-B–SEKDEL (pLTK-110) in soluble protein extracts from microtubers (*8*) of independent potato transformants. Leaves or microtubers from plants transformed with a vector that did not contain a LT-B coding sequence were used as a control (C). The total soluble protein in samples was measured with a Coomassie dye binding assay kit (Bio-Rad) with BSA as the standard. (**D**) Immunoprecipitation of rLT-B and rLT-B–SEKDEL from extracts of transgenic tobacco leaves. Immunoprecipitates with LT-B–specific antibodies of unboiled (−) samples and samples heated in a water bath at 95°C for 5 min (+) of radiolabeled tobacco leaves (*13*) were run on a 15% polyacrylamide gel (*15*). Plants expressing rLT-B (lanes 1 and 2), rLT-B–SEKDEL (lanes 3 and 4), and a plant transformed with a vector that did not contain the LT-B coding sequence (lanes 5 and 6) are shown. The prestained low molecular mass protein standards (Bio-Rad) are indicated on the left.

Table 1. Neutralization of *E. coli* enterotoxin activity by serum and mucosal extracts from mice orally immunized with transgenic tobacco leaf extracts. The assay was conducted with the use of mouse Y-1 adrenal cells in miniculture (*18*). Titer is defined as the reciprocal of the highest serum or mucosal extract dilution showing complete neutralization of biological activity of 50 pg of toxin. Values for serum and mucosal samples cannot be directly compared.

Antibody samples	Titer
Sera from mice orally inoculated with bacterially expressed rLT-B	320
Sera from mice orally inoculated with transgenic tobacco leaf extract	320
Mucosal extracts from mice orally inoculated with bacterially expressed rLT-B	5120
Mucosal extracts from mice orally inoculated with transgenic tobacco leaf extract	5120

tuber protein, representing 0.01% of total protein.

Immunoprecipitates from radioactively labeled leaves (*13*) of transformed tobacco plants were analyzed by SDS–polyacrylamide gel electrophoresis (PAGE). A protein band was observed in extracts from pLTB-110 plants (Fig. 1D) that comigrated with bacterial rLT-B monomer (*14*). A slightly larger peptide was observed in plants expressing rLT-B–SEKDEL. The quantity of LT-B monomer in both samples increased when the sample was boiled before SDS-PAGE, which suggests that the plant-derived antigens were at least partially oligomerized in plant cells in a fashion similar to bacterial rLT-B (*15*). We verified the presence of an oligomeric form of rLT-B–SEKDEL from leaves by gel filtration on Sephacryl 200. The antigen eluted with an apparent molecular mass of 38 kD, which is similar to that of bacterial rLT-B pentamer (*14*, *15*). Because the rLT-B–SEKDEL protein showed structural and binding properties similar to those of rLT-B expressed in *E. coli* and accumulated in significantly greater amounts in plants than did rLT-B, we used pLTK-110 plants for all immunogenicity studies.

To determine oral immunogenicity, BALB/c mice were given a crude soluble extract from pLTK-110 tobacco leaves (*16*) by gavage. Each dose had 12.5 μg of antigen [as determined by LT-B ELISA (*9*)] and was administered on days 0, 4, 21, and 25. Another group of mice was given 12.5 μg of purified *E. coli*–expressed rLT-B with the same gavage schedule. Serum and mucosal samples were collected on days 30 to 32, and antibody titers were examined by ELISA (*9*) for rLT-B–specific antibodies. Amounts of serum antibodies were similar in the two groups (Fig. 2), which indicates that the plant-derived material retained immunogenic properties of the bacterial rLT-B. Mucosal samples from mice immunized with tobacco rLT-B–SEKDEL also showed antibodies to LT-B. Toxin neutralization assays showed the protective nature of these antibodies; serum and mucosal immunoglobulins from animals immunized with plant or bacterially expressed antigen neutralized the biological activity of LT to the same extent (Table 1).

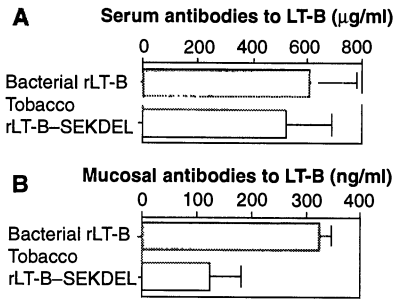

Fig. 2. Oral immunogenicity in BALB/c mice from extracts of transgenic tobacco leaves expressing rLT-B–SEKDEL or rLT-B expressed in *E. coli*. Mice were gavaged with solutions containing 12.5 μg of rLT-B on days 0, 4, 21, and 25. On day 28, animals were killed, and serum (**A**) and mucosal (**B**) materials were examined by ELISA for the presence of antibodies to LT-B (*9*).

Individual pLTK-110 potato transformants were clonally propagated and grown to maturity in soil. On the basis of ELISA, a feeding of 5 g of tuber (one oral dose) was calculated to deliver 15 to 20 μg of rLT-B–SEKDEL, which can be consumed by a mouse in 2 to 6 hours. Mice that consumed pLTK-110 potato samples developed serum IgG and mucosal IgA that were specific for LT-B (Fig. 3). These responses were compared to those of animals immunized with 20-μg doses of bacterial rLT-B by oral gavage. The immune response to the purified bacterial rLT-B was greater than to any of the transgenic potato samples. This may indicate that some factors in the plant limit or interfere with antigen reactivity with lymphoid tissue. Mice fed with the control (nontransformed) tubers developed no LT-B–specific antibodies in either serum or mucosal samples.

The fact that feeding mice transgenic potatoes expressing a recombinant antigen caused an oral immune response demonstrates the feasibility of using transgenic plants as expression and delivery systems for oral vaccines. We anticipate that an increase in recombinant protein concentration in the plant tissue will lead to an increased immune response. Other antigens expressed in plants may also act as oral immunogens (*17*); in separate studies (with Mary Estes of Baylor College of Medicine) we have found that the capsid protein of Norwalk virus can be expressed in either insect cells or plants, and it causes an oral immune response in mice. We conclude that there may be broad utility for transgenic plants in production and delivery of subunit vaccines.

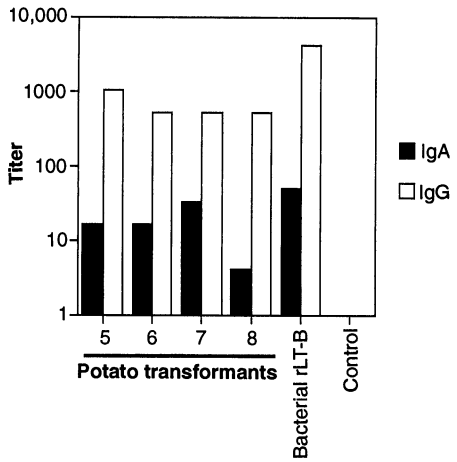

Fig. 3. Oral immunogenicity in BALB/c mice from potato tubers expressing rLT-B–SEKDEL. Mice were fed 5 g of transgenic tuber slices (transformants 5, 6, 7, and 8), control untransformed tuber slices (control), or 20 μg of bacterial rLT-B on days 0, 4, 14, and 18. On day 28, animals were killed, and serum and mucosal materials were examined by ELISA for the presence of antibodies to LT-B (*9*). Titer is defined as the reciprocal of the highest dilution to give an optical absorbance (OD 405 nm) ≥ 0.2.

REFERENCES AND NOTES

1. J. Mestecky and J. R. McGhee, *Curr. Top. Microbiol. Immunol.* **146**, 3 (1989); J. Holmgren, C. Czerkinsky, N. Lycke, A. M. Svennerholm, *Immunobiology* **184**, 157 (1992).
2. J. Holmgren, N. Lycke, C. Czerkinsky, *Vaccine* **11**, 1179 (1993); H. J. De Aizpurua and G. J. Russell-Jones, *J. Exp. Med.* **167**, 440 (1988).
3. R. B. Sack, *J. Infect. Dis.* **142**, 279 (1980).
4. T. K. Sixma *et al.*, *Nature* **351**, 371 (1991).
5. J. D. Clements and R. A. Finkelstein, *Infect. Immun.* **21**, 1036 (1978); J. Holmgren and A. M. Svennerholm, *Prog. Allergy* **33**, 106 (1983); J. D. Clemens *et al.*, *Lancet* **335**, 270 (1990); H. Peltola *et al.*, *ibid.* **338**, 1285 (1991).
6. A plant gene expression cassette, pIBT210, was constructed to contain a polylinker (Nco I, Bam HI, Sma I, Kpn I, and Sac I) positioned between a cauliflower mosaic virus (CaMV) 35S promoter–tobacco etch virus 5′-UTR (TEV leader) fusion [J. C. Carrington *et al.*, *Plant Cell* **3**, 953 (1991)] and the 3′ flank of the soybean *vspB* gene [H. S. Mason *et al.*, *ibid.* **5**, 241 (1993)] in the Hind III–Eco RI sites of pUC19. The translation initiation site of LT-B in pJC217 [J. D. Clements and S. El-Morshidy, *Infect. Immun.* **46**, 564 (1984)] was modified to have an Nco I site by the use of polymerase chain reaction to generate a 67–base pair fragment that was ligated with pIBT210 to give pLTB5. Complimentary oligonucleotides encod-

T. A. Haq, H. S. Mason, C. J. Arntzen, Plant Biotechnology Program, Albert B. Alkek Institute of Biosciences and Technology, Texas A&M University, 2121 Holcombe Boulevard, Houston, TX 77030, USA.
J. D. Clements, School of Medicine, Department of Microbiology and Immunology, Tulane Medical Center, 1430 Tulane Avenue, New Orleans, LA 70112, USA.
*To whom correspondence should be addressed.

ing the amino acids SEKDEL (Fig. 1A) were annealed and ligated at the Spe I site of the gene encoding LT-B in pJC217 to obtain pLTK217. The LT-B coding regions from pJC217 and pLTK217 were subcloned into pBluescript KS (Stratagene) at Eco RI–Hind III, then excised with Bam HI–Dra I and ligated into pIBT210 to form pLTB200 and pLTK200, respectively. The Sac I fragments of pLTB200 and pLTK200 were ligated into pLTB5–Sac I to give pLTB210 and pLTK210. The Hind III–Eco RI cassettes in pLTB210 and pLTK210 were transferred to the *Agrobacterium* T-DNA vector pBI101 [R. A. Jefferson *et al.*, *EMBO J.* **13**, 3901 (1987)] to give pLTB110 and pLTK110.

7. R. Horsch *et al.*, *Plant Molecular Biology Manual*, S. B. Gelvin, R. A. Schilperoort, D. P. S. Verma, Eds. (Kluwer Academic, Dordrecht, Netherlands, 1991), pp. A5, 1–A5, 9. H. Wenzler, G. Mignery, G. May, W. Park, *Plant Sci.* **63**, 79 (1989).
8. A. Perl, D. Aviv, L. Willmitzer, E. Galum, *Plant Sci.* **73**, 87 (1991).
9. L. Cardenas and J. D. Clements, *Infect. Immun.* **61**, 4629 (1993). Leaf or tuber samples extracted with PBST [10 mM sodium phosphate buffer (pH 7.2), 105 mM NaCl, and 0.05% Tween 20] containing 40 mM sodium ascorbate, 20 mM EDTA, and 1 mM phenylmethylsulfonyl fluoride (PMSF) were cleared at 12,000*g*, diluted with PBST, and loaded in duplicate wells. ELISA was as described.
10. J. D. G. Jones *et al.*, *EMBO J.* **4**, 2411 (1985).
11. S. Munro and H. R. B. Pelham, *Cell* **48**, 899 (1987); C. Wandelt *et al.*, *Plant J.* **2**, 181 (1992). Single-letter abbreviations for the amino acid residues are as follows: D, Asp; E, Glu; K, Lys; L, Leu; and S, Ser.
12. T. R. Hirst and J. Holmgren, *Proc. Natl. Acad. Sci. U.S.A.* **84**, 7418 (1987).
13. Tobacco leaves were excised from plants, radiolabeled by uptake of ^{35}S-labeled methionine and cysteine (EXPRE ^{35}S^{35}S protein labeling mix, NEN/Dupont) through the petiole, and homogenized in a tissue grinder in buffer [50 mM tris (pH 7.5), 0.2 M NaCl, 2 mM EDTA, 2 mM PMSF, 5 mM dithiothreitol, 0.1% bovine serum albumin (BSA), and 1% Triton X-100]. The extracts were centrifuged at 15,000*g* for 10 min, and the supernatant was immunoprecipitated as in H. S. Mason *et al.*, *Plant Mol. Biol.* **11**, 845 (1988) with the use of goat antibody to LT-B. The supernatant was eluted in Laemmli sample buffer [U. K. Laemmli, *Nature* **227**, 680 (1970)] at room temperature and separated by SDS-PAGE. The gel was fluorographed [W. M. Bonner and R. A. Laskey, *Eur. J. Biochem.* **46**, 83 (1974)].
14. T. A. Haq, H. S. Mason, J. D. Clements, C. J. Arntzen, data not shown.
15. S. J. S. Hardy *et al.*, *Proc. Natl. Acad. Sci. U.S.A.* **85**, 7901 (1988).
16. Tobacco leaves were frozen and ground to powder in liquid nitrogen in a Waring blender. The soluble protein was extracted (*9*) and cleared at 15,000*g*. The fraction precipitating between 40 and 60% $(NH_4)_2SO_4$ saturation was resuspended in phosphate-buffered saline for oral immunization.
17. H. S. Mason, D. M. Lam, C. J. Arntzen, *Proc. Natl. Acad. Sci. U.S.A.* **89**, 11745 (1992); C. J. Arntzen *et al.*, *Vaccines '94, Modern Approaches to New Vaccines Including Prevention of AIDS*, F. Brown, R. M. Chanock, H. S. Ginsberg, R. A. Lerner, Eds. (Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 1994), pp. 339–344.
18. D. A. Sack and R. B. Sack, *Infect. Immun.* **11**, 334 (1975).
19. Supported in part by NIH grant 1 R01 A136519-01 (J.D.C. and C.J.A.) and by the Texas Advanced Technologies Program, project 999902-084 (H.S.M.).

18 January 1995; accepted 14 April 1995

Generation and Assembly of Secretory Antibodies in Plants

Julian K.-C. Ma,* Andrew Hiatt, Mich Hein, Nicholas D. Vine, Fei Wang, Paul Stabila, Craig van Dolleweerd, Keith Mostov, Thomas Lehner

Four transgenic *Nicotiana tabacum* plants were generated that expressed a murine monoclonal antibody kappa chain, a hybrid immunoglobulin A-G heavy chain, a murine joining chain, and a rabbit secretory component, respectively. Successive sexual crosses between these plants and filial recombinants resulted in plants that expressed all four protein chains simultaneously. These chains were assembled into a functional, high molecular weight secretory immunoglobulin that recognized the native streptococcal antigen I/II cell surface adhesion molecule. In plants, single cells are able to assemble secretory antibodies, whereas two different cell types are required in mammals. Transgenic plants may be suitable for large-scale production of recombinant secretory immunoglobulin A for passive mucosal immunotherapy. Plant cells also possess the requisite mechanisms for assembly and expression of other complex recombinant protein molecules.

Secretory immunoglobulin A (SIgA) is the most abundant form of immunoglobulin (Ig) in mucosal secretions, where it forms part of the first line of defense against infectious agents. The molecule exists mainly in the 11*S* dimeric form, in which two monomeric IgA antibody units are associated with the small polypeptide joining (J) chain and with a fourth polypeptide, secretory component (SC). The ability to produce monoclonal SIgA would be of substantial value, but the synthesis is complicated because it requires plasma cells secreting dimeric IgA (dIgA) as well as epithelial cells expressing the polymeric Ig receptor (pIgR). Normally, pIgR on the epithelial basolateral surface binds dIgA, initiating a process of endocytosis, transcytosis, phosphorylation, proteolysis, and ultimate release of the SIgA complex at the apical surface into the secretion (*1*). Here, we focused on the ability of transgenic plants to assemble secretory antibodies.

Genes encoding the heavy and light chains of a murine antibody (Guy's 13), a murine J chain, and a rabbit SC were introduced into separate transgenic tobacco plants. Guy's 13 is a murine IgG1 monoclonal antibody (mAb) that recognizes the streptococcal antigen (SA) I/II cell surface adhesion molecule of *Streptococcus mutans* and *S. sobrinus* (*2*). Transgenic full-length Guy's 13 has been generated in *N. tabacum* plants and was found to be correctly assembled (*3*). Modification of the heavy chain by replacement of its Cγ3 domain with Cα2 and Cα3 domains from an IgA-secreting hybridoma (MOPC 315) did not affect the assembly or function of the antibody (IgA-G) produced in transgenic plants (*3*). Protein immunoblot analysis (*4*) of the IgA-G plant extract with antiserum to the κ light chain under nonreducing conditions showed a band of ~210 kD, which is consistent with the presence of the extra constant region domains in the IgA-G antibody construct as compared with the original IgG1 antibody (Fig. 1A, lanes 1 and 3). A number of smaller proteolytic fragments were also detected, which is consistent with previous findings (*3*). A mouse J chain construct that consisted of coding-length complementary DNA (cDNA) was amplified with synthetic oligonucleotide primers corresponding to the NH_2-terminal MKTHLL and the COOH-terminal SCYPD sequences of the mouse J chain (*5*). The SC construct used in this study consisted of coding-length cDNA amplified with synthetic oligonucleotide primers corresponding to the NH_2-terminal MALFLL sequence and the AVQSAE sequence near the COOH-terminus of rabbit pIgR (*6*). Transgenic plants were then regenerated (*7*).

The plants that expressed the J chain were crossed with those expressing IgA-G. The progeny showed a second major Ig band at ~400 kD, approximately twice the relative molecular mass of the IgA-G molecule (Fig. 1A, lane 4), which suggested that a dimeric antibody (dIgA-G) had been assembled. Mature plants that expressed dIgA-G were crossed with a homozygous plant that expressed SC. The progeny plants (SIgA-G) included those that produced a higher mo-

J. K.-C. Ma, N. D. Vine, C. van Dolleweerd, T. Lehner, Department of Immunology, United Medical and Dental Schools, Guy's Hospital, 28th Floor, Guy's Tower, St. Thomas's Street, London SE1 9RT, UK.
A. Hiatt, Planet Biotechnology, 8445 Camino Santa Fe, Suite 102, San Diego, CA 92121, USA.
M. Hein, F. Wang, P. Stabila, Department of Cell Biology, Division of Plant Biology, Scripps Research Institute, 10666 North Torrey Pines Road, La Jolla, CA 92037, USA.
K. Mostov, Department of Anatomy, School of Medicine, University of California, San Francisco, CA 94143, USA.
*To whom correspondence should be addressed.