

No endogenous A β production in presenilin-deficient fibroblasts

To the editor — Presenilins (PSs) are involved in the cleavage of integral membrane proteins, such as amyloid- β precursor protein (APP), Notch, ErbB-4 and CD44. The question as to whether or not they are proteases is therefore relevant for the broad field of cell biological research. Recently, Armogida *et al.* reported that small amounts of the A β amyloid peptide can be detected in mouse PS-deficient fibroblasts¹. This result challenged the conclusions of previous publications^{2,3}. However, the assay used by Armogida *et al.* is not specific for mouse A β , and we were not able to reproduce their findings using a mouse A β -specific ELISA assay (Fig. 1a). When we used a similar immune precipitation/western blotting

assay as Armogida *et al.*¹, we did however detect trace amounts of A β peptide, even in the unconditioned culture medium (Fig. 1b). This assay is critically dependent on the monoclonal antibody, W0-2, which was raised against a specific human A β epitope. As both rabbit and bovine A β , but not mouse A β , are identical to human A β , they will be equally and efficiently detected by the W0-2 antibody. Thus, bovine A β from the serum added to the culture medium or rabbit A β (see accompanying letter by Grimm *et al.*) in the sera used to immunoprecipitate A β are potential sources of false-positive results. Although it is possible to incubate fibroblasts for brief periods in serum-free media (see accompanying

letter by Petit *et al.*), it remains uncertain whether the bovine A β that accumulated before can be completely depleted from the cells. In any event, the affinity of the W0-2 antibody is too low to detect mouse A β from cells that overexpress mouse APP in this assay (Fig. 1c), demonstrating unequivocally that the A β detected in the experiments of Armogida *et al.* cannot be of mouse origin. Thus, the claim that PS-deficient fibroblasts secrete A β is not supported by the published data. A more extensive pdf report will be made available on request.

Omar Nyabi*, **Stefan Pype†**, **Marc Mercken‡**, **An Herreman***, **Paul Saftig‡**, **Katleen Craessaerts***, **Lutgarde Serneels***, **Wim Annaert*** and **Bart De Strooper*§**

*Center for human genetics, KULeuven and Flanders interuniversity institute for Biotechnology (VIB), Herestraat 49, 3000 Leuven, Belgium

†Johnson & Johnson Pharmaceutical Research & Development, Beerse, Belgium

‡University of Kiel, Institute of Biochemistry, Olshausenstr. 40, D-24098 Kiel, Germany

§Bart.destrooper@med.kuleuven.ac.be

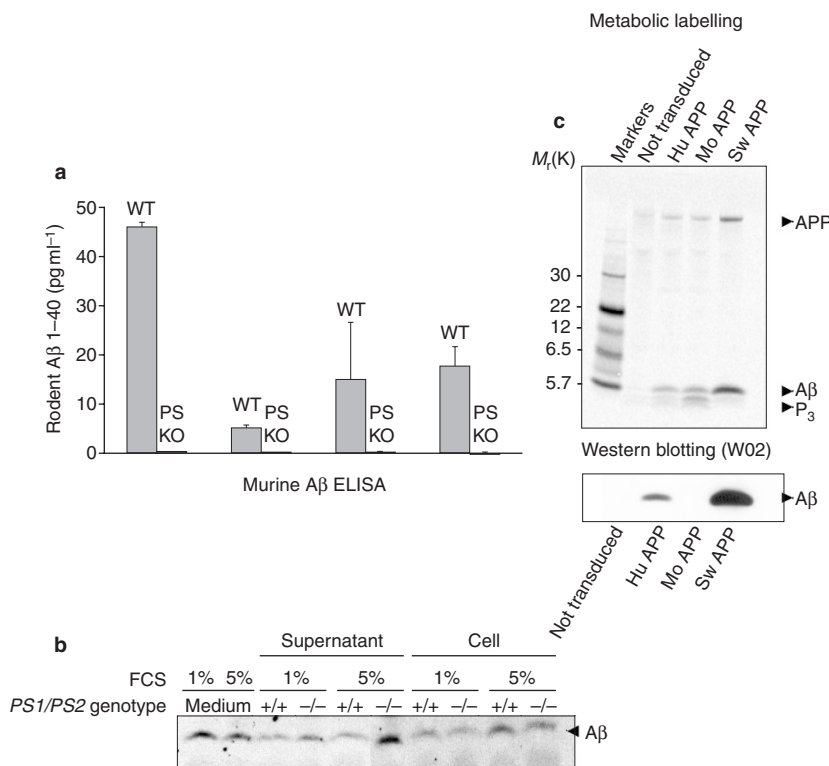


Figure 1 Presenilin-deficient fibroblasts do not synthesize A β . **a**, Culture media of wild-type (WT) and PS-deficient (PSKO) fibroblasts were assayed for the presence of rodent A β in four independent experiments. No A β was detected in the PSKO cells. **b**, Next, total A β was immunoprecipitated from the unconditioned medium, the conditioned culture medium (supernatant) and the cell extracts, and detected by western blotting using the W02 monoclonal antibody, as described by Armogida *et al.*¹. Positive signals were detected in all lanes, including unconditioned medium, probably reflecting the presence of bovine or rabbit A β . **c**, Finally, neuronal cells were transfected with Semliki Forest Virus (SFV) coding for human APP (Hu APP), mouse APP (Mo APP) or human APP containing the Swedish mutation (Sw APP). Cells were metabolically labelled, and A β was immunoprecipitated and visualized by autoradiography (top). The same blot was then probed with W02. Although human A β is clearly detected, mouse A β is not (bottom).

Potential external source of A β in biological samples

To the editor — Armogida *et al.*¹ reported production of the A β amyloid peptide by mouse fibroblasts that were devoid of PSs and concluded that PSs are not essential for A β production. In this study, A β was detected by western blotting with the W02 antibody after immunoprecipitation with polyclonal antibodies. Drs Checler and De Strooper kindly provided us with the original antibodies and cells to test this hypothesis. Reproducing their experiments, we detected identical levels of A β in conditioned media, irrespective of the PS genotype (Fig. 2a).

We assumed that A β may have been derived from three sources: the fibroblasts,

the polyclonal antisera or the foetal calf serum (FCS; see accompanying letter by Nyabi *et al.*). Indeed, we found that A β is present in the polyclonal antisera (Fig. 2b). Accordingly, we failed to detect A β as soon as the two external sources were excluded from the cell culture experiments (Fig. 2c). Thus, we conclude that under the conditions we used, the western blot antibody W02 does not detect mouse A β . This is supported by the observation that the W02 epitope in mice is different from that in humans. We further conclude that all A β detected in the mouse fibroblast-conditioned media was most likely derived from polyclonal antibodies and FCS. Therefore, it seems that Armogida *et al.* have encountered the same situation and detected external rabbit and bovine A β , but not mouse fibroblast A β .

Although it has gone unreported that A β may be introduced in samples by the A β antibody, we want to point out that this problem may not be specific to the polyclonal antibodies we have tested, but could be a more general phenomenon. In combination with a highly sensitive detection assay, we suggest that special controls should be included to eliminate potential external sources from the experiment.

Marcus O.W. Grimm*, **Inge Tomic***
and **Tobias Hartmann*†**

*Zentrum für Molekulare Biologie of Heidelberg,
University of Heidelberg, Im Neuenheimer Feld
282, 69120 Heidelberg, Germany

†e-mail: Tobias.Hartmann@mail.zmbh.uni-heidelberg.de

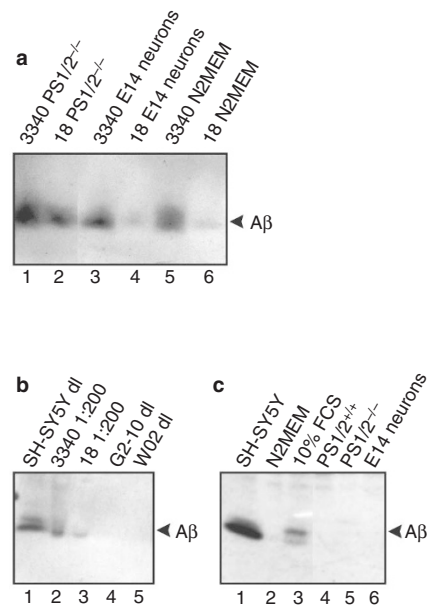


Figure 2 Antibodies and FCS contaminate samples with A β . **a**, Detection of A β from mouse cell conditioned media (CM) is dependent on the presence of rabbit polyclonal antisera and FCS. 1.5 ml of the indicated CM (lanes 1–4) and non-conditioned media (NCM; lanes 5,6) were immunoprecipitated with FCA3340 and FCA18 antisera, as used by Armogida *et al.*¹. Lanes 1,2 mouse fibroblast CM with FCS; lanes 3,4, CM-N2 minimal essential medium (MEM) without FCS; lanes 5,6, NCM. **b**, A β is present in polyclonal antisera. CM and antibodies were directly loaded (dl) or diluted 1:200 in N2MEM. Lane1, positive control SH-SY5Y human neuroblastoma cell line CM (1.2 μ l); lanes 2–5, antibody analysis. Dilution of antibodies did not prevent A β detection. **c**, W02 (refs 4,5) does not detect mouse A β . Immunoprecipitation of CM and NCM with anti-A β antibody G2-10 at 5 μ g ml⁻¹. Lane 1, human SH-SY5Y CM; lane 2, N2MEM-NCM without FCS; lane 3, DMEM-NCM with 10% FCS, G2-10 precipitates the low A β levels from FCS; lanes 4,5, mouse fibroblast (wild-type and presenilin-deficient) CM with reduced FCS content; lane 6, mouse mixed cortical neurons N2MEM-CM (without FCS). Importantly, A β was not detectable in absence of polyclonal antisera or FCS from mouse CM. All western blots were probed with the W02 antibody⁵.

Reply — We recently found that cleavage of APP and Notch could be discriminated pharmacologically with novel non-peptidic inhibitors⁶ and that the endogenous production of secreted and intracellular A β 40 and A β 42 peptides were not affected by PS deficiency in fibroblasts¹. This challenges the view that PS could function as the genuine γ -secretase. In the accompanying letters, Nyabi *et al.* and Grimm *et al.* suggest that our data could be a result of artifactual detection of contaminating bovine or rabbit A β present in the culture medium or in the FCA antisera used in our previous study¹.

Here, we carried out other experiments with and without fibroblasts (Fig. 3a) performed under strictly identical conditions (150-mm dishes, same volume, same batch of serum, identical percentage of serum, identical immunoprecipitation/western blot procedures and same times of ECL revelation). We fully confirmed the detection of A β in the medium of PS^{+/+} and PS-deficient cells (Fig. 3a), but not in the medium

alone, even at concentrations of up to 10% foetal calf serum (FCS; see Fig. 3a). Analyses of five independent experiments indicated a small and statistically insignificant decrease in secreted A β recoveries (Fig. 3d). Furthermore, we examined the production of endogenous secreted and intracellular A β from PS^{+/+} and PS^{-/-} fibroblasts cultured with a synthetic substitute of serum devoid of any molecule from animal or human origin (Fig. 3b). Under these conditions, an identical production of FCA3340-immunoprecipitable A β was observed in the secretion media and lysates of both PS^{+/+} and PS^{-/-} cell systems (Fig. 3b,d).

We also examined the possibility that A β contamination could be derived from immunoprecipitating FCA rabbit antisera themselves. Surprisingly, we found that FCA3340 antiserum, and to a much lesser extent FCA18 antiserum, contained immunoreactivity that was detectable with the anti-A β W02 monoclonal antibody when antisera were loaded directly on the

gels, whereas nothing was detectable with the FCA3542 antiserum (data not shown). It is unlikely that this endogenous rabbit A β present in FCA3340 antiserum accounted for the FCA3340-immunoprecipitable A β observed with various PS-containing cells as A β -immunoreactivity was not detected after immunoprecipitation of medium alone with FCA3340 antiserum (Fig. 3a). This agrees with the observations of Nyabi *et al.*, where the authors claim that mice neurons do not produce FCA3340-precipitable A β (Fig. 1c, bottom). As the FCA3340 serum batch is identical to the one used in our lab, these authors should also have detected putative contaminating rabbit A β in their immunoprecipitation/western blot procedure.

However, to definitively rule out any possibility of contamination by either bovine or rabbit A β , we performed experiments in serum free-Prolifex medium and performed immunoprecipitations with the IgG-purified fraction of FCA3340 and FCA18 antisera. Again, the IgG-purified

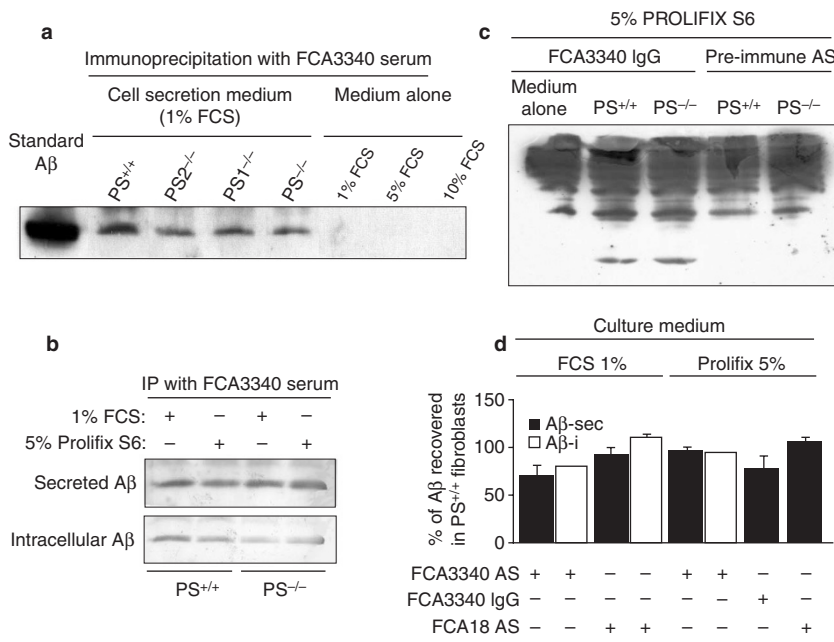


Figure 3 A β secretion by PS^{+/+}, PS1^{-/-}, PS2^{-/-} and PS^{-/-} cells. 150-mm dishes of fibroblasts of indicated and fully confirmed¹ PS genotype were cultured in 1% FCS (**a,b**) or 5% Prolifix S6 (**b,c**) then secreted (**a-c**) or intracellular A β (**b**) was precipitated with FCA3340 antiserum (**a,b**), IgG-purified fraction of FCA3340 (**c**), or pre-immune serum (**c**) before analysis by western blot with the WO2 antibody, as described¹. Identical procedures were performed on medium alone (**a,c**) containing the indicated percentage of foetal calf serum (**a**) or Prolifix (**c**). Note the lack of FCA3340-precipitable A β -like-immunoreactivity in medium alone under all experimental conditions, even at 10% FCS. In **a**, A β immunoreactivities in PS^{-/-}, PS1^{-/-} and PS2^{-/-} were 68%, 77% and 74% of that recovered in PS^{+/+}, respectively. In **d**, the quantification of secreted (A β -sec, black bars) or intracellular (A β -i, white bars) A β recovered with PS-deficient cells cultured in the indicated medium conditions and immunoprecipitated with the indicated antibody source is shown. Bars are expressed as the percentage (taken as 100) of A β recovered in identical conditions with PS^{+/+} fibroblasts and are the means \pm SEM of 3–5 (black bars) or 2 (white bars) independent experiments. Fibroblasts cell cultures, A β secretion conditions, immunoprecipitation with FCA3340, western blot with WO2, and 16.5% Tris-tricine gel analyses were performed as described¹, except that 150-mm dishes were used instead of 100-mm dishes to favour the immunoprecipitation of putative contaminating A β . IgG-purified fractions of FCA antisera were prepared as previously described¹⁰. Experiments carried out with synthetic substitute of serum devoid of any animal or human proteins (ProlifixS6; Biomedica, Boussons, France) were performed as above.

fraction of FCA3340 (Fig. 3c) and FCA18 (data not shown) precipitated A β from PS^{-/-} and PS^{+/+} cells to the same extent (Fig. 3d), whereas nothing was observed under identical conditions in absence of cells (Fig. 3c, (Prolifix) medium alone) or with pre-immune serum (Fig. 3c). Finally, we used western blotting to determine whether WO2 labelling of human A β could be displaced by a peptide mimicking the murine epitope. In two distinct experiments, we found

that a 40% inhibition was consistently observed with 1 mM murine peptide (corresponding to A β 4–9), whereas the A β 11–21 peptide did not cause any inhibition (data not shown). The IC₅₀ for the human A β 4–9 peptide is approximately 0.02 mM. Therefore, the murine peptide can also compete in this assay (that is, where human A β is denatured), although with a 50-fold lower potency than that triggered by the human sequence.

Altogether, we confirm that endogenous secreted and intracellular A β production by fibroblasts is independent of the PS content and that, at least in fibroblasts, there exists a PS-independent activity responsible for endogenous A β production. The fact that a γ -secretase-like activity is indeed detectable in PS^{-/-} cells is further demonstrated in a recent paper⁷. This study shows that a Notch signalling pathway exists in PS^{-/-} cells that does not result in Notch intracellular domain (NICD) production, but which is blocked by γ -secretase inhibitors⁷. This is in agreement with our paper showing that although A β remains unaffected in PS^{-/-} cells, NICD production is fully impaired¹. This is another demonstration that in cells devoid of PSs, there is/are catalytic activity(ies) that are sensitive to inhibitors, the pharmacological spectrum of which is a typical γ -secretase inhibition. That several PS-dependent and PS-independent γ -secretase activities could occur is also demonstrated by the groups of Drs De Strooper and Saftig⁸. Thus, it seems that various cleavages taking place at the 42nd position of A β can be discriminated by the absence or presence of PS, as A β 42 was reported to be affected by a PS1 deficiency⁹ whereas A β 2–42 remained virtually unaffected⁸. As far as one considers that the carboxy-terminal ends of all A β species (either truncated or not at their amino terminus) are generated by γ -secretase, this is another demonstration that γ -secretase-derived A β species with distinct susceptibility to PS exist in PS1^{-/-} fibroblasts.

Agnes Petit*, Bruno Vincent*, Sabine Scarzello*, Marianna Armogida, Cristine Alves da Costa* and Frédéric Checler*

IPMC du CNRS, UMR6097, 660 route des Lucioles, Sophia-Antipolis, 06560, Valbonne, France
*e-mail: checler@ipmc.cnrs.fr

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