No endogenous Aβ 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 Aβ amyloid peptide can be detected in mouse PS-deficient fibroblasts[1]. This result challenged the conclusions of previous publications[2,3]. However, the assay used by Armogida *et al.*[1] is not specific for mouse Aβ, and we were not able to reproduce their findings using a mouse Aβ-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 Aβ peptide, even in the unconditioned culture medium (Fig. 1b). This assay is critically dependent on the monoclonal antibody, WO-2, which was raised against a specific human Aβ epitope. As both rabbit and bovine Aβ, but not mouse Aβ, are identical to human Aβ, they will be equally and efficiently detected by the WO-2 antibody. Thus, bovine Aβ from the serum added to the culture medium or rabbit Aβ (see accompanying letter by Grimm *et al.*) in the sera used to immunoprecipitate Aβ 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 Aβ that accumulated before can be completely depleted from the cells. In any event, the affinity of the WO-2 antibody is too low to detect mouse Aβ from cells that overexpress mouse APP in this assay (Fig. 1c), demonstrating unequivocally that the Aβ detected in the experiments of Armogida *et al.* cannot be of mouse origin. Thus, the claim that PS-deficient fibroblasts secrete Aβ is not supported by the published data. A more extensive pdf report will be made available on request.

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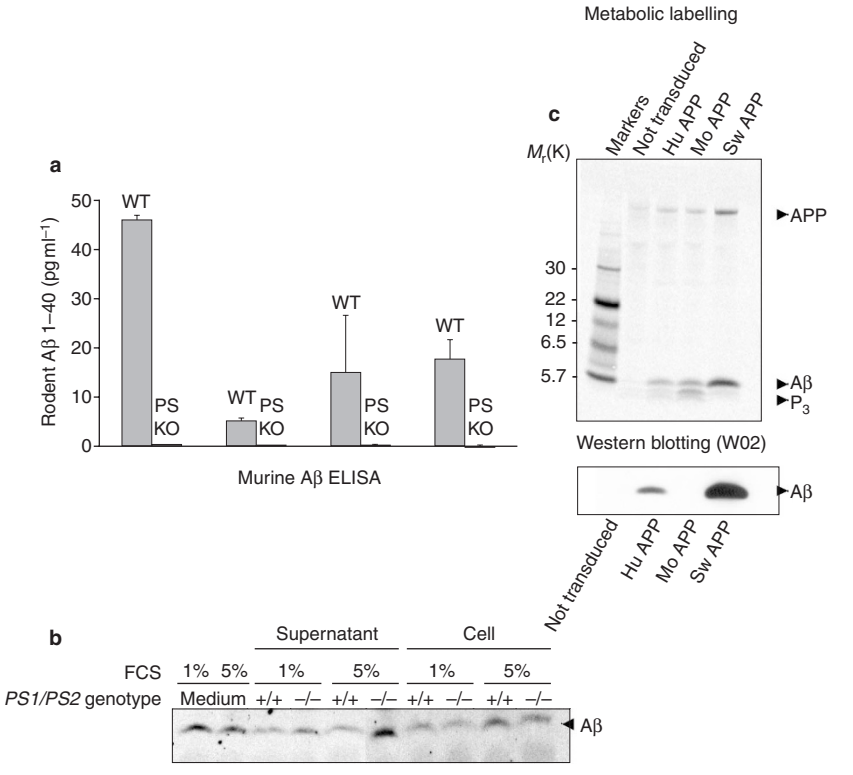

Figure 1 **Presenilin-deficient fibroblasts do not synthesize Aβ. a**, Culture media of wild-type (WT) and PS-deficient (PSKO) fibroblasts were assayed for the presence of rodent Aβ in four independent experiments. No Aβ was detected in the PSKO cells. **b**, Next, total Aβ was immunoprecipitated from the unconditioned medium, the conditioned culture medium (supernatant) and the cell extracts, and detected by western blotting using the WO2 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 Aβ. **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 Aβ was immunoprecipitated and visualized by autoradiography (top). The same blot was then probed with WO2. Although human Aβ is clearly detected, mouse Aβ is not (bottom).

Potential external source of Aβ in biological samples

To the editor — Armogida *et al.*[1] reported production of the Aβ amyloid peptide by mouse fibroblasts that were devoid of PSs and concluded that PSs are not essential for Aβ production. In this study, Aβ 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 Aβ in conditioned media, irrespective of the PS genotype (Fig. 2a).

We assumed that Aβ 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 Aβ is present in the polyclonal antisera (Fig. 2b). Accordingly, we failed to detect Aβ 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 Aβ. This is supported by the observation that the W02 epitope in mice is different from that in humans. We further conclude that all Aβ 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 Aβ, but not mouse fibroblast Aβ.

Although it has gone unreported that Aβ may be introduced in samples by the Aβ 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.

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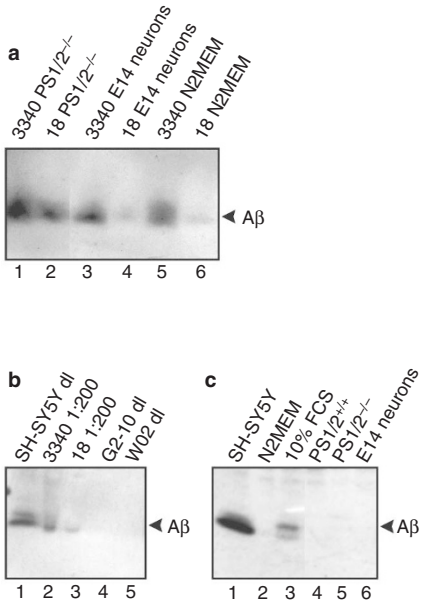

Figure 2 **Antibodies and FCS contaminate samples with Aβ. a**, Detection of Aβ from mouse cell conditioned media (CM) is dependent on the presence of rabbit polyclonal antisera and FCS. 1.5 ml of the indicated CM (lanes 1–4) and non-conditioned media (NCM; lanes 5,6) were immunoprecipitated with FCA3340 and FCA18 antisera, as used by Armogida *et al.*[1]. Lanes 1,2 mouse fibroblast CM with FCS; lanes 3,4, CM-N2 miminal essential medium (MEM) without FCS; lanes 5,6, NCM. **b**, Aβ 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 (12 μl); lanes 2–5, antibody analysis. Dilution of antibodies did not prevent Aβ detection. **c**, W02 (refs 4,5) does not detect mouse Aβ. Immunoprecipitation of CM and NCM with anti-Aβ antibody G2-10 at 5 μg 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 Aβ 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, Aβ was not detectable in absence of polyclonal antisera or FCS from mouse CM. All western blots were probed with the W02 antibody[5].

Reply — We recently found that cleavage of APP and Notch could be discriminated pharmacologically with novel non-peptidic inhibitors[6] and that the endogenous production of secreted and intracellular Aβ40 and Aβ42 peptides were not affected by PS deficiency in fibroblasts[1]. 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 Aβ present in the culture medium or in the FCA antisera used in our previous study[1].

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 Aβ 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 Aβ recoveries (Fig. 3d). Furthermore, we examined the production of endogenous secreted and intracellular Aβ 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 Aβ was observed in the secretion media and lysates of both $PS^{+/+}$ and $PS^{-/-}$ cell systems (Fig. 3b,d).

We also examined the possibility that Aβ 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-Aβ 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 Aβ present in FCA3340 antiserum accounted for the FCA3340-immunoprecipitable Aβ observed with various PS-containing cells as Aβ-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 Aβ (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 Aβ in their immunoprecipitation/western blot procedure.

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

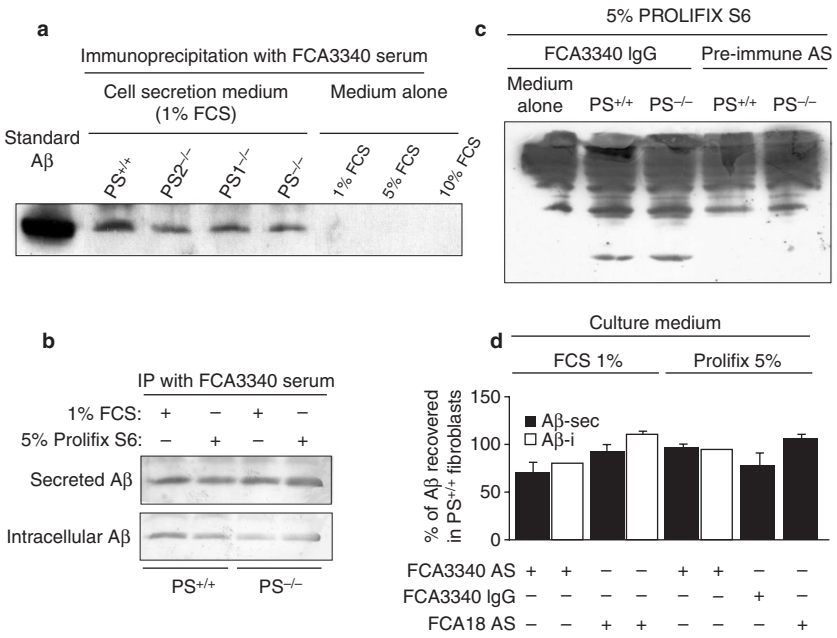

Figure 3 **Aβ secretion by PS$^{+/+}$, PS1$^{-/-}$, PS2$^{-/-}$ and PS$^{-/-}$ cells.** 150-mm dishes of fibroblasts of indicated and fully confirmed[1] *PS* genotype were cultured in 1% FCS (**a**,**b**) or 5% Prolifix S6 (**b**,**c**) then secreted (**a**–**c**) or intracellular Aβ (**b**) was precipitated 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 described[1]. 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 Aβ-like-immunoreactivity in medium alone under all experimental conditions, even at 10% FCS. In **a**, Aβ immunoreactivities in PS$^{-/-}$, PS1$^{-/-}$ and PS2$^{-/-}$ were 68%, 77% and 74% of that recovered in PS$^{+/+}$, respectively. In **d**, the quantification of secreted (Aβ-sec, black bars) or intracellular (Aβ-i, white bars) Aβ 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 Aβ recovered in identical conditions with PS$^{+/+}$ fibroblasts and are the means ± SEM of 3–5 (black bars) or 2 (white bars) independent experiments. Fibroblasts cell cultures, Aβ secretion conditions, immunoprecipitation with FCA3340, western blot with WO2, and 16.5% Tris-tricine gel analyses were performed as described[1], except that 150-mm dishes were used instead of 100-mm dishes to favour the immunoprecipitation of putative contaminating Aβ. IgG-purified fractions of FCA antisera were prepared as previously described[10]. Experiments carried out with synthetic substitute of serum devoid of any animal or human proteins (ProlifixS6; Biomedia, Boussens, France) were performed as above.

fraction of FCA3340 (Fig. 3c) and FCA18 (data not shown) precipitated Aβ 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 Aβ 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 Aβ4–9), whereas the Aβ11–21 peptide did not cause any inhibition (data not shown). The IC_{50} for the human Aβ4–9 peptide is approximately 0.02 mM. Therefore, the murine peptide can also compete in this assay (that is, where human Aβ is denatured), although with a 50-fold lower potency than that triggered by the human sequence.

Altogether, we confirm that endogenous secreted and intracellular Aβ production by fibroblasts is independent of the PS content and that, at least in fibroblasts, there exists a PS-independent activity responsible for endogenous Aβ production. The fact that a γ-secretase-like activity is indeed detectable in PS$^{-/-}$ cells is further demonstrated in a recent paper[7]. 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 inhibitors[7]. This is in agreement with our paper showing that although Aβ remains unaffected in PS$^{-/-}$ cells, NICD production is fully impaired[1]. 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 Saftig[8]. Thus, it seems that various cleavages taking place at the 42nd position of Aβ can be discriminated by the absence or presence of PS, as Aβ42 was reported to be affected by a PS1 deficiency[9] whereas Aβ2-42 remained virtually unaffected[8]. As far as one considers that the carboxy-terminal ends of all Aβ species (either truncated or not at their amino terminus) are generated by γ-secretase, this is another demonstration that γ-secretase-derived Aβ species with distinct susceptibility to PS exist in PS1$^{-/-}$ fibroblasts.

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