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# Induced Pluripotent Stem Cells Generated from Patients with ALS Can Be Differentiated into Motor Neurons

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The generation of pluripotent stem cells from an individual patient would enable the large-scale production of the cell types affected by that patient's disease. These cells could in turn be used for disease modeling, drug discovery, and eventually autologous cell replacement therapies. Although recent studies have demonstrated the reprogramming of human fibroblasts to a pluripotent state, it remains unclear whether these induced pluripotent stem (iPS) cells can be produced directly from elderly patients with chronic disease. We have generated iPS cells from an 82-year-old woman diagnosed with a familial form of amyotrophic lateral sclerosis (ALS). These patient-specific iPS cells possess properties of embryonic stem cells and were successfully directed to differentiate into motor neurons, the cell type destroyed in ALS.

**A**myotrophic lateral sclerosis (ALS) is a neurodegenerative disorder in which motor neuron loss in the spinal cord and motor cortex leads to progressive paralysis and death (1). Studies aimed at understanding the root causes of motor neuron death in ALS and efforts to develop new therapeutics would be greatly advanced if a robust supply of human motor neurons carrying the genes responsible for this condition could be generated. It was recently reported that mouse (2–5) and human (6) skin fibroblasts can be reprogrammed to a pluripotent state, similar to that of an embryonic stem (ES) cell, following transduction with retroviruses encoding *KLF4*, *SOX2*, *OCT4*, and *c-MYC*. However, it remains unclear whether induced pluripotent stem (iPS) cells can be generated directly from elderly patients with chronic disease—that is, from material that has been exposed to disease-causing agents for a lifetime—and whether such patient-specific iPS cells could be differentiated into the particular cell types that would be needed to treat or study the patient's condition.

Here, we show that iPS cells can be produced using skin fibroblasts collected from an 82-year-old patient diagnosed with a familial form of ALS. These patient-specific iPS cells

possess a gene expression signature similar to that of human ES cells and can be differentiated into cell types representative of each of the three embryonic germ layers. We have used these iPS cells to produce patient-specific motor neurons and glia, the cell types implicated in ALS pathology.

Under human research subject and stem cell protocols approved by the institutional review boards and embryonic stem cell research oversight committees of both Harvard University and Columbia University, we recruited patients with ALS and healthy controls to donate skin biopsies to be used in reprogramming studies and the production of pluripotent stem cell lines. Our initial studies focused on two female Caucasian siblings, patients A29 and A30, who were 82 and 89 years old at the time of donation. These sisters are both heterozygous for the same rare L144F (Leu<sup>144</sup> → Phe) dominant allele of the superoxide dismutase (*SOD1*) gene that is associated with a slowly progressing form of ALS (7). Patient A29 had a clear clinical manifestation of motor neuron disease, including difficulty in swallowing and weakness of the arms and legs. Patient A30 was clinically asymptomatic but had signs of upper motor neuron disease upon physical examination, presenting with bilateral plantar responses and hyperreflexia. These sisters are among the oldest living patients with disease-associated *SOD1* alleles.

Primary skin cells isolated by biopsy from these patients exhibited the morphology (Fig. 1A), cell cycle profile (fig. S2), and antigenic expression pattern (fig. S3) of human fibroblasts. Transgenes encoding *KLF4*, *SOX2*, *OCT4*, and *c-MYC* were introduced into these fibroblasts by means of vesicular stomatitis virus glycoprotein (VSVg)-pseudotyped Moloney-based retroviruses. About 30,000 fibroblasts were transduced twice over 72 hours, cultured for 4 days in standard fibroblast medium, and then passaged onto a feeder layer of mouse embryonic fibroblasts in an ES cell-supportive medium. As described previously,

within 1 week hundreds of colonies composed of rapidly dividing cells with a granular morphology not characteristic of ES cells had appeared (6). However, 2 weeks later, a small number of colonies with an ES cell morphology (Fig. 1, B and C) could be identified. All ES cell-like colonies, 12 from A29 and three from A30, were chosen by hand and clonally expanded. Of these colonies, seven from A29 and one from A30 gave rise to stable cell lines that could be further expanded. Because donor A29 had been diagnosed with classical ALS, we focused our initial characterization on three putative patient-specific iPS cell lines derived from her.

To verify that the patient-specific iPS cell lines were genetically matched to the donor, we performed DNA fingerprinting analysis of the three putative iPS cell lines (A29a, A29b, and A29c) and the fibroblasts from which they were derived. Allele assignments indicated that each of the putative iPS cell lines carried the genotype of the patient's fibroblasts (table S1). Additionally, we used direct sequencing (Fig. 1D) and an allele-specific restriction fragment length polymorphism (fig. S1, A and B) to compare the *SOD1* genotype of these cell lines with that of the donated fibroblasts and genotyping results in the patient's medical history. In each of these assays, we detected the expected L144F polymorphism in both the putative A29 iPS cell lines and the fibroblasts from which they were derived, but not in fibroblasts isolated from a healthy control individual (A18). Furthermore, polymerase chain reaction (PCR) analysis of genomic DNA from these three cell lines revealed that they all carried integrated copies of the four retroviral transgenes with which they had been transduced (fig. S1C).

To establish that reprogramming of the patient fibroblasts had occurred and that the putative iPS cells were pluripotent, we evaluated their similarity to ES cells. Like ES cells (8)—and unlike the parental A29 fibroblasts—the A29 iPS cells displayed an active cell cycle profile, with 35% of cells in S or G<sub>2</sub>/M phases (fig. S2). The putative iPS cell lines also maintained a normal karyotype (fig. S1D). Additionally, all three iPS cell lines exhibited strong alkaline phosphatase activity and expressed several ES cell-associated antigens (SSEA-3, SSEA-4, TRA1-60, TRA1-81, and NANOG), but were not immunoreactive for a fibroblast-associated antigen (TE-7) (Fig. 1, E and F, and fig. S3).

Quantitative reverse transcription PCR showed that genes expressed in pluripotent cells (*REX1*/*ZFP42*, *FOXD3*, *TERT*, *NANOG*, and *CRIP1*/*TDGF1*) were transcribed at levels comparable to human ES cells in each of the three putative iPS cell lines (Fig. 2A). Moreover, the stem cell marker genes *SOX2* and *OCT4* were not expressed in the patient fibroblasts, whereas the endogenous loci in the putative iPS cells had become activated to levels similar to those in ES cells (Fig. 2B). As in previous reports (6), expression levels from the endogenous *KLF4* and *c-MYC* loci were similar in ES cells, iPS cells, and the parental fibroblasts

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(Fig. 2B). Human iPS cells have been shown in some (6), but not all (6, 9), cases to silence expression of the retroviral transgenes used to re-program them. RT-PCR analyses performed using primers specific to the retroviral transcripts demonstrated nearly complete silencing of viral *SOX2* and *KLF4*. However, some expression of viral *OCT4* and *c-MYC* persisted, as previously reported (6).

Pluripotent cells are by definition capable of differentiating into cell types derived from each of the three embryonic germ layers (10). A property of both ES cells and previously established human iPS cells is their ability, when plated in suspension culture, to form embryoid bodies (EBs) composed of diverse cell types (fig. S4A) (6, 9, 10). When grown in these conditions, all three iPS cell lines from patient A29 readily formed EBs (Fig. 3A). Immunocytochemical analyses of EBs

after 13 to 16 days of culture showed that each line had spontaneously differentiated into cell types representative of the three embryonic germ layers (Fig. 3, B to F, and fig. S4B). Together, these data indicate that we have reprogrammed primary fibroblasts isolated from an elderly ALS patient into iPS cells.

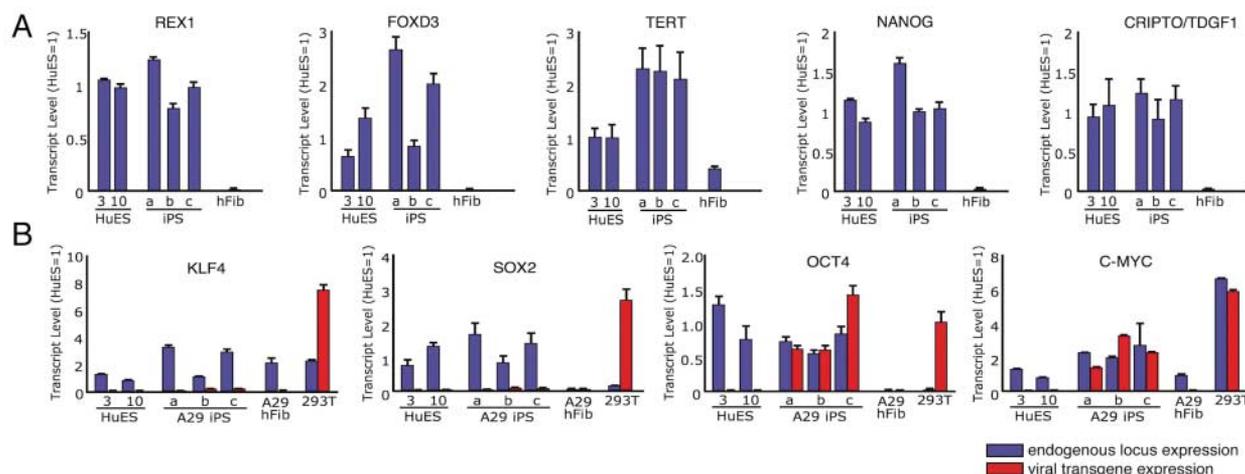
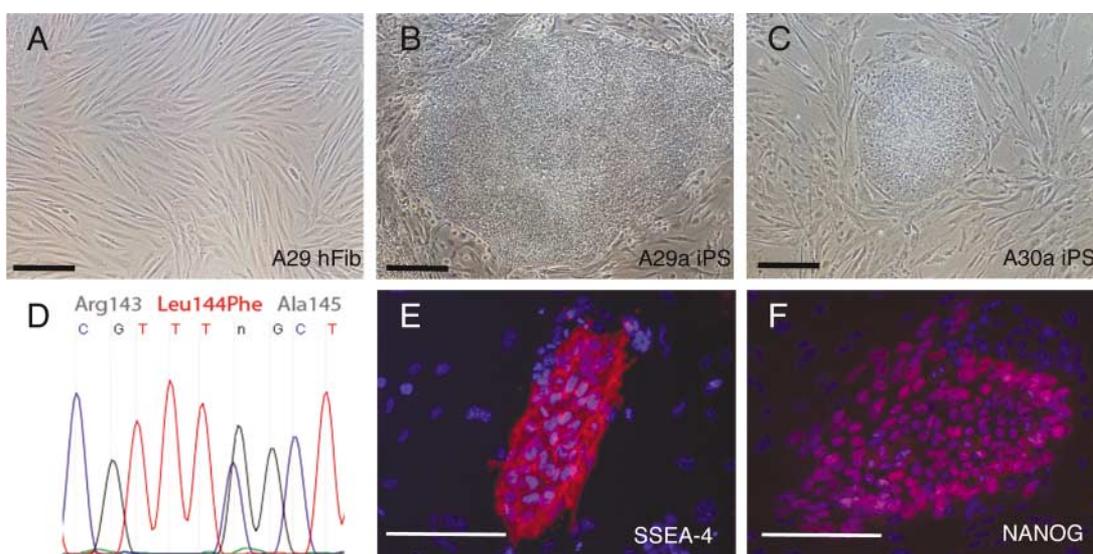
Much of the hope invested in patient-specific stem cells is based on the assumption that it will be possible to differentiate them into disease-relevant cell types. ALS is characterized by the progressive degeneration of spinal cord motor neurons (1, 11), and recent studies have shown that both cell-autonomous and non-cell-autonomous factors contribute to disease progression (12, 13). In particular, glia from ALS animal models were shown to produce factors that are toxic to motor neurons (14–16). These studies indicate that pro-

duction of both motor neurons and glia would be essential for mechanistic studies and perhaps for eventual cell replacement therapies for ALS.

We therefore attempted to generate spinal motor neurons and glia with the use of a directed differentiation protocol developed for mouse and human ES cells (17–20). EBs formed from iPS cells were treated with two small molecules: an agonist of the sonic hedgehog (SHH) signaling pathway and retinoic acid (RA) (fig. S5A). When these differentiated EBs were allowed to adhere to a laminin-coated surface, neuron-like outgrowths were observed (Fig. 4A). Many of these processes stained positive for a neuronal form of tubulin,  $\beta$ -tubulin IIIb (TuJ1), confirming their neuronal nature (Fig. 4B and fig. S6).

To further characterize the cells after directed differentiation, we plated dissociated EBs onto

**Fig. 1.** iPS cells can be established from patient fibroblasts after biopsy. (A) Primary dermal fibroblasts (hFib, human fibroblasts) derived from an 82-year-old female ALS patient, A29. (B) iPS cells produced from patient A29. (C) iPS cells produced from a second patient, A30, sister to patient A29. (D) Direct sequencing of a PCR product from A29 iPS cells, confirming the presence of one copy of the dominant L144F *SOD1* allele. (E and F) SSEA-4 and NANOG protein expression in A29 iPS cells. Scale bars, 200  $\mu$ m.



**Fig. 2.** A29 iPS cells are similar to human ES cells in their expression of genes associated with pluripotency. (A) The ES cell–associated transcripts *REX1/ZFP42*, *FOXD3*, *TERT*, *NANOG*, and *CRIPTO/TDGF1* are activated in the three putative iPS cell lines (A29a, A29b, and A29c) to levels comparable to human ES cells, as measured by quantitative RT-PCR. (B) Primers specific for either endogenously (blue) or virally (red) encoded transcripts of the four reprogramming factors

were used to measure their respective expression levels. Expression was detected from all four endogenous loci in the iPS cells at levels similar to those in the human ES cell lines HuES-3 and HuES-10. Expression from the retroviral *KLF4* and *SOX2* transgenes was not detected, although both retroviral *OCT4* and *c-MYC* were expressed. As a positive control for expression of the viral transgenes, 293T cells were transiently transfected with the four plasmids used to produce virus.

laminin-coated slides as a single-cell suspension. TuJ1-positive neurons that coexpressed the motor neuron marker HB9 [a motor neuron-specific transcription factor (17)] could be readily identified in cultures derived from both the A29a and A29b cell lines (Fig. 4C and figs. S5B and S7). In cultures differentiated from A29b iPS cells, we examined 3262 nuclei (from three independent differentiation experiments) and found that 651 stained for HB9, indicating that 20% of all cells expressed this motor neuron marker. Moreover, more than 90% of these HB9-positive cells also expressed ISLET 1/2 [ISL, transcription factors involved in motor neuron development (17, 18)]

(Fig. 4, E to H, and figs. S5C and S8). More than half of these HB9- and ISL-positive neurons expressed choline acetyltransferase (ChAT), demonstrating an advanced degree of cholinergic motor neuron maturation (17) (figs. S5D and S9B). Cells expressing the spinal cord progenitor markers OLIG2 and PAX6 were also prevalent in these cultures (fig. S9A), which suggests that these patient-specific iPS-derived motor neurons arose from progenitors similar to those found in the developing spinal cord. In addition, cells expressing the glial markers GFAP (glial fibrillary acidic protein) and S100 were readily identified (Fig. 4D and fig. S10). Thus, patient-specific iPS cells—like

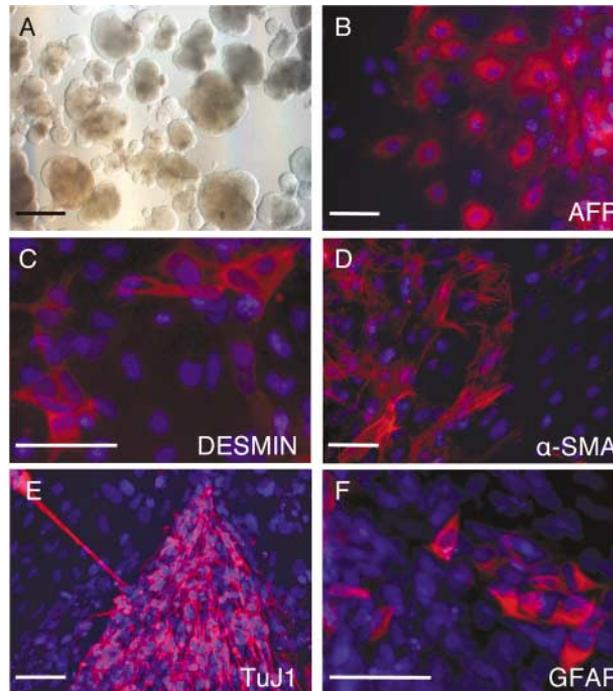
human ES cells—can respond appropriately to developmentally relevant patterning signals, demonstrating the feasibility of leveraging the self-renewal of iPS cells to generate a potentially limitless supply of the cells specifically affected by ALS.

Our results with patient-derived cells confirm the initial finding that the exogenous expression of only four factors—*KLF4*, *SOX2*, *OCT4*, and *c-MYC*—is sufficient to reprogram human fibroblasts to a pluripotent state (6). Previous reports using these four genes to generate human iPS cells have required the overexpression of either a murine viral receptor (6) or additional oncogenes such as *Large T Antigen* and *TERT* (21). In contrast, our results using retroviruses pseudotyped to transduce human cells dispel the suggestion by a recent study that these four genes are not sufficient to induce reprogramming (21).

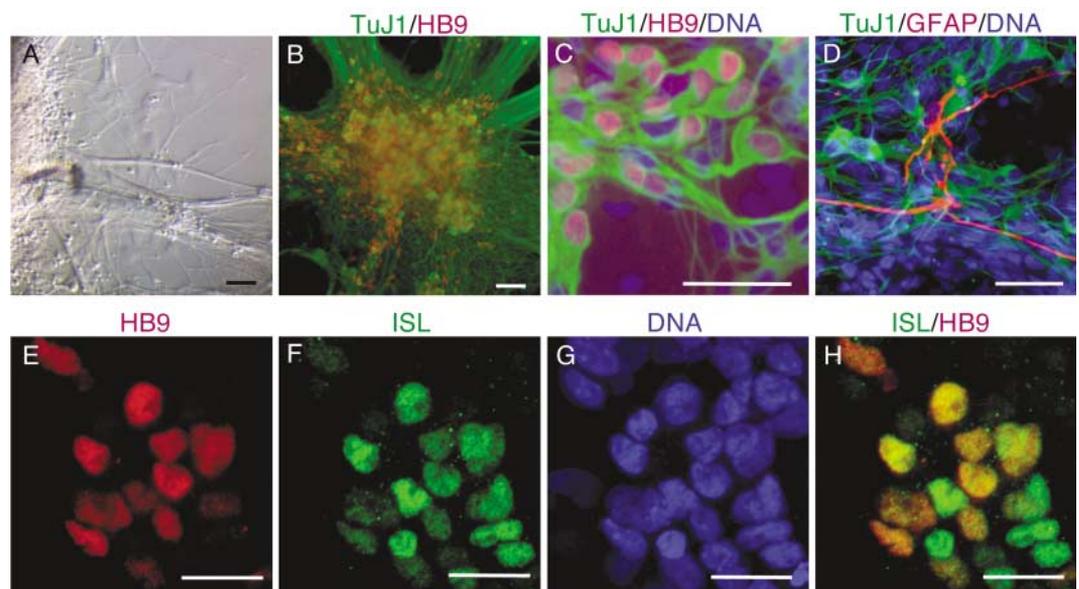
We have demonstrated that it is possible to produce patient-specific pluripotent stem cells. It is particularly encouraging that neither the advanced age nor the severely disabling disease of patient A29 prevented us from reprogramming her fibroblasts. Attempts to generate similar pluripotent cell lines using somatic cell nuclear transfer and ES cell fusion have been confronted by technical, logistical, and political obstacles that have not yet been overcome (22, 23). The use of defined reprogramming factors for the generation of patient-specific iPS cells has allowed us to circumvent these obstacles. Note that the multiple integrations of retroviral DNA in the host genome, which were required for reprogramming, did not preclude our ability to terminally differentiate these cells into motor neurons. Nonetheless, long-term studies will be needed to compare the in vitro physiology of iPS-derived motor neurons and those derived from human ES cell lines.

Our study also demonstrates the feasibility of producing large numbers of motor neurons with a

**Fig. 3.** Patient-specific iPS cells are pluripotent stem cells. (A) EBs formed from A29b iPS cells, 5 days after seeding. Scale bar, 200  $\mu$ m. (B to F) These EBs contained cells representative of each of the three embryonic germ layers: endoderm [(B),  $\alpha$ -fetoprotein (AFP)], mesoderm [(C), desmin; (D),  $\alpha$ -smooth muscle actin ( $\alpha$ -SMA)], and ectoderm [(E),  $\beta$ -tubulin IIIb (TuJ1); (F), GFAP]. Scale bars, 100  $\mu$ m.



**Fig. 4.** iPS cells generated from ALS patients can be differentiated into motor neurons. A29b iPS cell EBs were patterned with RA and SHH, then plated on laminin, either whole (A and B) or after dissociation (C to H), and allowed to mature for 7 to 15 days. (A) Neuron-like outgrowths are visible from whole A29b patient-specific iPS cell EBs. (B) Extensive TuJ1-positive neuronal processes grow out from plated whole iPS EBs, which contain a high proportion of HB9-stained nuclei. (C) Neuronal identity of HB9-expressing cells is confirmed by high-magnification image of HB9 and TuJ1 coexpression in dissociated patient-specific motor neuron cultures. (D) GFAP-expressing glial cells can be found in addition to TuJ1-expressing neurons in differentiated patient-specific iPS cell cultures. [(E) to (H)] The motor neuron identity of HB9- and TuJ1-positive cells is confirmed by the coexpression of HB9 and ISL. HB9 (E) and ISL (F) localization is nuclear (G) and highly coincident (H). Scale bars, 100  $\mu$ m [(A) to (D)], 75  $\mu$ m [(E) to (H)].



patient's exact genotype, which would be immune-matched to that individual—a long-sought goal of regenerative medicine. However, several major challenges must be resolved before cell replacement therapy using iPS technology can become a clinical reality. First, among several other safety issues, iPS-derived neurons will not be suitable for transplantation until the oncogenic genes and retroviruses (24, 25) used here are replaced with more controlled methods of reprogramming. Second, it likely will be necessary to understand and correct any intrinsic defects in the patient's neurons and glia before they can be rationally used as a basis for cell therapy.

Many recent insights into the pathophysiology of ALS come from the study of familial forms of this disease. The patient-specific iPS cells produced here will be important tools for further studies of mechanisms by which familial disease arises. However, more than 90% of ALS patients are afflicted by a sporadic form of disease, thought to arise from complex interactions between genetic and environmental factors (26). As a result of these complexities, it has been impossible until now to devise in vitro cell-based models for this most common type of ALS. Patient-specific iPS cells generated from individuals with sporadic disease would carry the precise constellation of genetic information associated with pathology in that person. This approach would allow study of

living motor neurons generated from ALS cases with unknown genetic lesions, providing insight into their intrinsic survival properties, their interactions with other cell types, and their susceptibility to the environmental conditions that are considered to play an important role in ALS pathogenesis.

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#### Supporting Online Material

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Materials and Methods

Figs. S1 to S10

Tables S1 and S2

References

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## Amyloid- $\beta$ Dynamics Correlate with Neurological Status in the Injured Human Brain

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The amyloid- $\beta$  peptide ( $A\beta$ ) plays a central pathophysiological role in Alzheimer's disease, but little is known about the concentration and dynamics of this secreted peptide in the extracellular space of the human brain. We used intracerebral microdialysis to obtain serial brain interstitial fluid (ISF) samples in 18 patients who were undergoing invasive intracranial monitoring after acute brain injury. We found a strong positive correlation between changes in brain ISF  $A\beta$  concentrations and neurological status, with  $A\beta$  concentrations increasing as neurological status improved and falling when neurological status declined. Brain ISF  $A\beta$  concentrations were also lower when other cerebral physiological and metabolic abnormalities reflected depressed neuronal function. Such dynamics fit well with the hypothesis that neuronal activity regulates extracellular  $A\beta$  concentration.

**A** $\beta$  is the principal constituent of the hallmark amyloid plaques found in Alzheimer's disease and is the target of many potential treatments for the disease (1). However, little is known about the concentration and dynamics of this secreted peptide in the extracellular space of the human brain where these plaques form. In vitro and animal studies have shown that neuronal and synaptic activity dynamically regulate soluble extracellular  $A\beta$  concentrations (2–4). Whether similar regulation of  $A\beta$  levels occurs in the human brain is unknown.

We used intracerebral microdialysis (5) to obtain serial brain interstitial fluid (ISF) samples in 18 intensive care unit (ICU) patients who had sustained acute brain injury and were undergoing invasive intracranial monitoring for clinical purposes. In all patients,  $A\beta_{1-42}$  was detected in hourly or bihourly intracranial microdialysis samples. None had a diagnosis of Alzheimer's disease or dementia, demonstrating that  $A\beta$  is a normal constituent of human brain extracellular fluid (6). The  $A\beta_{1-42}$  enzyme-linked immunosorbent assay (ELISA) used detects  $A\beta$  species from amino acid 1 to amino acid 28 or greater (3, 7).

There were rising trends in brain ISF  $A\beta$  concentrations over several hours to days in most patients, though the specific pattern of these trends was variable (Fig. 1, B, D, and F; Fig. 2B; and Fig. 4, A to D). Median brain ISF  $A\beta_{1-42}$  at 60 to 72 hours was 59% higher than at 0 to 12 hours (Fig. 1G) ( $P = 0.0002$ , Wilcoxon signed rank test). Urea concentrations in the same samples, which control for the stability of the microdialysis catheter function (8), remained stable over the same time frame (Fig. 1H) (median 14% lower,  $P = 0.06$ , Wilcoxon signed rank test). Thus, the observed  $A\beta$  dynamics are likely to be of cerebral origin and not an artifact of the measurement procedure.

$A\beta_{1-42}$  concentrations were lower in microdialysate than in concomitantly sampled ventricular cerebrospinal fluid (CSF) (Fig. 2, B and D). However, at the flow rate used (0.3  $\mu$ l/min), the microdialysate is not in complete equilibrium with the surrounding extracellular space (5). To calculate the true brain extracellular concentrations,

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