Do amniotic-fluid derived stem cells differentiate into neurons in vitro?

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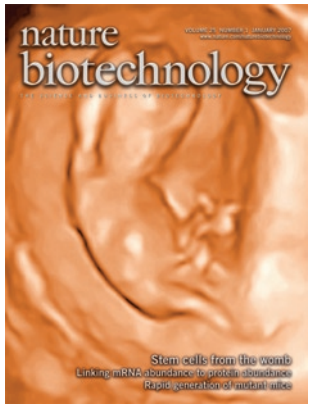
To the editor:

Last year, De Coppi *et al.*[1] reported that amniotic fluid–derived stem (AFS) cells can yield differentiated cells that express lineage-specific markers and acquire characteristic functions *in vitro*. In particular, in addition to other cell types, they assert that AFS cells can give rise *in vitro* to neurons. To support this, the authors provide data indicating that AFS cells exposed to a medium that has been previously shown to promote neuronal differentiation express typical neuronal markers. They also show that they can record a barium-sensitive potassium current from these cells and that the cells release glutamate. Given our specific competence and experience, we cannot comment on the characterization of nonneuronal types. We do think, however, that the presented data do not provide compelling evidence that AFS cells can specifically differentiate into neurons.



In this respect, several aspects of the results obtained by De Coppi *et al.* are of concern. First, the authors cite nestin as a marker "first defined in neural stem cells." But nestin is not only expressed in neuroepithelial stem cells, but also in mesonephric mesenchyme, endothelial cells of developing blood vessels and kidney cells (see ref. 2 and references therein). In addition, in Figure 3a of the De Coppi *et al.* paper, the cytoskeletal staining of the intermediate filament nestin appears to be weak and atypical of the usual intracellular distribution of nestin.

Second, in our view, Figure 3b provides insufficient support for the claim that "individual cells (have) pyramidal morphology" (and does not confirm that the cells shown are neurons). The authors mention that the differentiation medium used has been shown previously to bias differentiation toward dopaminergic neurons[3]. However, no attempts are made at analyzing any of the markers related to the dopaminergic lineage (including a very obvious one, tyrosine hydroxylase).

Third, GIRK2 gene expression (Fig. 3c in the paper) cannot be considered a specific neuronal marker. GIRK (G-protein regulated inward-rectifier potassium) channels are hetero- or homotetrameric assemblies of different Kir3.0 and Kir3.2 subunits. The Kir3.2 subunit (also termed GIRK2) possesses at least four isoforms that arise from differential splicing including Kir3.2a, Kir3.2b, Kir3.2c and Kir3.2d[4–7]. Kir3.2 isoforms are widely distributed in different cell types and are not specific to neurons; in fact, Kir3.2b (GIRK2b) is considered to be ubiquitously expressed and Kir3.2d (GIRK2d) is expressed also in mouse testis[6–8].

Fourth, by definition, GIRK channels are inward-rectifier potassium channels activated by G proteins following interaction between specific G proteins and agonists. In the absence of agonist, the background activity of GIRK channels is negligible, as clearly demonstrated by dose-response experiments (e.g., see refs. 9,10). The currents observed in the absence of agonist in Figure 3d of the De Coppi *et al.* paper suggest that a channel other than GIRK is active in these cells. It is possible that the agonist-independent currents result from activation of IRK (inward-rectifier potassium) channels, which are expressed in many cells types in addition to being expressed in neurons[11].

Fifth, barium caused partial depression of current in the AFS-derived cells (Fig. 3e of the De Coppi *et al.* paper). Barium, however, is a nonspecific blocker of different K^+ channel subtypes, including GIRK, IRK, K2p and some Kv channels[12]. These experiments cannot provide evidence that AFS cells develop into functional and active neurons.

And lastly, Figure 3f of their paper shows glutamate release from the AFS cells following growth in the differentiation medium. Glutamate can be released from astrocytes, osteoblasts, liver cells and neurons, and, as such, glutamate release is not a neuron-specific property[13–15].

The main feature that distinguishes neurons from other cell types is their ability to fire tetrodotoxin-sensitive action potentials with a characteristic shape and duration. To support the claim that AFS cells can differentiate into neurons, the authors could have used well-accepted approaches, such as determining whether (i) the cells express voltage-gated Na^+ channels, (ii) the cells have a resting membrane potential close to values expected for excitable cells and below the voltage for Na^+ channel steady-state inactivation, (iii) channel activation elicits tetrodotoxin-sensitive Na^+ current and (iv) the generation of sizeable Na^+ currents correlates with the capability of the cells to produce action potentials. One more critical parameter for the identification of neurons from undifferentiated stem cells could be the demonstration of synapses by electron microscopy. This is another hard marker of neuron identity not observed in $Tuj1^+$ neuron-like populations obtained from nonneural sources.

De Coppi *et al.* also present initial studies suggesting that AFS cells directed toward a neural lineage are able to widely engraft into the developing mouse brain (Fig. 4a–f from their paper). To us, the pictures presented only indicate that the authors are able to localize the donor cells within the choroid plexus and in close proximity to the ventricles using an antibody that recognizes the donor human cells. No claims about integration can be raised on the basis of the data presented. In addition, the author made no attempt to show that donor cells develop characteristic neuronal markers or

morphology. Indeed, considering that donor cells have been exclusively visualized by an antibody against a 65-kDa mitochondrial protein with perinuclear localization, it is difficult to understand the claim that transplanted cells "integrate seamlessly and appear morphologically indistinguishable from surrounding murine cells," which were only labelled by the nuclear stain DAPI (4'-6-diamidino-2-phenylindole).

In conclusion, in our view the article by De Coppi *et al.* fails to provide any convincing evidence to support the claim that AFS cells are able to generate neurons. Given the medical and ethical impact of such claims, it is important that the contention that AFS cells can generate functional neurons be fully substantiated.

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Cesar C Santos, Mark E Furth, Evan Y Snyder, Paolo De Coppi, Shay Soker & Anthony Atala respond:

We thank Toselli *et al.* for their comments on the neuronal differentiation of AFS cells. The goal of our report in *Nature Biotechnology* was to demonstrate that stem cells isolated from amniotic fluid are capable of self-renewal and of differentiation toward lineage pathways corresponding to the three embryonic germ layers[1]. Accordingly, we provided evidence that multiple, independent isolates of human AFS cells "were able to differentiate along adipogenic, osteogenic, myogenic, endothelial, neurogenic and hepatic pathways." Within this group, the early neurogenic pathway served as the example for the ability to yield cells derived in development from the ectoderm. We further used retroviral marking to conclude that the multipotentiality of AFS cells with respect to the three germ layers is a clonal property.

Although we have observed the expression by induced AFS cells of markers consistent with the neuroectodermal lineage, including genes important for the generation of dopamine neurons, we agree that the findings to date do not show the production of mature neurons. The data in our report represent work in progress toward that end. Indeed, we sought to distinguish in our language between the generation of a mature, differentiated cell type and corresponding tissue (e.g., osteoblasts and bone) and the expression of markers and functions consistent with earlier stages of cell differentiation (e.g., hepatocytic or neuronal lineage). Below, we respond to the detailed concerns of Toselli *et al.* regarding the ability of AFS cells to differentiate into neurons *in vitro*.

First, Toselli *et al.* are correct that expression of nestin is not restricted to neuroepithelial progenitor cells. In our paper, we showed the expression of nestin by AFS cells induced under two conditions found previously to bias to neurectodermal differentiation and of GIRK2 mRNA (which they mention as a third concern) under conditions that promote the differentiation of embryonic stem (ES) cells toward dopaminergic neurons. We believe that the intensity of staining and the intracellular distribution of nestin, primarily in intermediate filaments, that we have observed repeatedly in putative neural progenitors derived from AFS cells are indeed comparable to what has been reported in the literature for neural stem cells obtained from the central nervous system.

Second, we did not claim that the cells shown in Figure 3b of our paper were functional neurons. To date, we have not observed differentiation of AFS cells to a mature neuronal phenotype. We will present elsewhere evidence for the expression of a variety of lineage markers consistent with the potential to progress in this direction, including class III beta-tubulin (β-Tub III) and the microtubule-associated protein 2 (MAP2)[2–9]. We also have observed expression in induced cells of transcription factors important for the development of dopaminergic neurons, including Nurr1, pitx3 and Lmx1a[10–18]. We continue to seek conditions that promote robust expression of features of a mature neuronal phenotype, including action potentials, and specifically of dopaminergic neurons.

As noted by Toselli *et al.*, the expression of GIRK2 and the presence of a barium-sensitive potassium current do not define cells as neurons. However, the electrophysiological protocol employed has been used extensively in characterizing GIRK2 channels in neurons in hippocampus and also dopamine cells in substantia nigra and the ventral tegmental area of the brain[19]. The demonstration of a GIRK2-like current after *in vitro* differentiation is consistent with the potential for AFS cells to develop toward a neuronal phenotype. As suggested, there remains the necessity to demonstrate expression and functional capacity of voltage-sensitive sodium channels before cells generated from AFS cells can be identified as functional neurons[20].

Toselli *et al.* also point out that glutamate release is not neuron specific and can occur in astrocytes, osteoblasts and liver cells. The conditions used in the experiments showing glutamate release were based on previous literature reporting neural lineage differentiation of various multipotent stem cells and differ substantially from conditions we found to promote osteogenic or hepatogenic differentiation of AFS cells or reported to do so for other stem cells. A distinction between an astrocyte-like or a neuron-like phenotype would not alter the conclusion that AFS cells can be induced to differentiate along a neuroectodermal lineage.

The intracranial transplant experiments reported in our paper were in keeping with the overall aim of showing that AFS cells can yield derivatives representing each of the three germ layers. A first obvious goal of the transplantation study was to rule out the formation of tumors. This was accomplished. Second, we wished to determine whether the cells could intermix with and behave similarly to endogenous neural progenitors in a neural germinal zone over the period of time observed, achieving at least one threshold to support the entrance of AFS cell derivatives into a neuroectodermal lineage. When implanted into the cerebral ventricles

at birth, donor human AFS cells did, in fact, distribute throughout the periventricular germinal zone. The donor cells intermixed with endogenous neural progenitors and began to migrate, in concert with endogenous cells, along established migratory routes to appropriate targets, for example, the olfactory bulb. The observed migration of AFS-derived cells is not a general property of human cells implanted into the developing mouse brain but reflects lineage-appropriate behavior. By contrast, human fibroblasts transplanted at birth into the cerebral ventricles of mice fail to migrate and largely die[21]. The detailed characterization of fates of AFS cells implanted into the brain remains a work in progress.

In summary, we agree that it remains to be proven that AFS cells can differentiate to yield mature neurons and did not make such a claim in our published report. We believe that the evidence presented supports the conclusion that AFS cells are capable of entering the neuroectodermal lineage. The generation of action potentials and the assessment of the therapeutic potential of AFS-derived cells in animal models of neurodegeneration represent future goals. Together with evidence for the generation of lineages corresponding to endoderm and mesoderm, the data support the potential of AFS cell derivatives to enter the three fundamental embryonic germ layers. We also showed that AFS cells are capable of extensive self-renewal. Amniotic fluid has long been known to contain heterogeneous fetal cells of widely differing lineages and degrees of maturation. The population in amniotic fluid includes stem cells that may have washed out from the embryo at its early developmental stages, though they are more mature than those from the inner cell mass. The ability to reproducibly recover these AFS cells by a one-step immunoselection procedure from material that otherwise would be discarded offers a potentially valuable resource for exploring basic and clinical applications.

COMPETING INTERESTS STATEMENT

The authors declare competing financial interests: details accompany the full-text HTML version of the paper at http://www.nature.com/naturebiotechnology/.

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