FGF2, but not EGF, induces multiciliated ependymal cells to dedifferentiate and adopt radial glial features *in vitro*

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Abstract

Multiciliated ependymal cells form an epithelium lining most of the ventricular cavities of vertebrates brain. Although considered postmitotic and completely differentiated, ependymal cells maintain some phenotypic characteristics of neural stem cells. Thereby, under specific conditions they behave as neural stem cells, developing radial glia characteristics, and undergoing asymmetric division. Our group is searching for factors that promote **dedifferentiation of ependymal cells** in vitro. We developed a simple method to obtain pure cultures of non-adherent multiciliated ependymal cells from **adult rats**. These cultures were used to investigate the effect of FGF2 on the differentiation state and the aggregation of ependymal cells. Thus, FGF2 treated ependymal cells lose cilia and hence mobility, and after 7 days they aggregate to form irregular spheres (diameter ≥ 20-30 µm). Such changes were not observed when EFG was used instead of FGF2. To assess the specificity of FGF2 action on cell aggregation, the FGF receptor inhibitor PD166866 and an anti-FGF2 neutralizing antibody were used. In both conditions the aggregative effect of FGF2 was abolished. No cell proliferation was observed during sphere formation, at least in such experimental conditions. Spheres were analyzed by immunocytochemistry using radial glia markers. They were positive for GFAP, vimentin, BLBP and GLAST. These data suggest that FGF2 promotes the identity loss in multiciliated ependymal cells in vitro, which are transformed into cells with radial glia features.

Introduction

Ependymal cells cover most of the ventricular cavities and the central canal of the spinal cord of the vertebrate nervous system. They form a simple cuboidal epithelium that separates the cerebrospinal fluid (CSF) and the nervous parenchyma. Ependymal cells form part of the adult neurogenic niche in the subventricular zone (SVZ) (Doetsch et al. 1997) where they are in intimate contact with the neural stem cells (NSC) and their progeny. Recent findings demonstrate that ependymal cells are quiescent in normal conditions, and so they do not fulfil the defining criteria of stem cells (Carlen et al. 2009). However, several evidences suggest that they may behave as NSC under specific circumstances, such as nervous tissue injury (Namiki and Tator 1999), stroke (Carlen et al. 2009; Zhang et al. 2007), carcinogenic transformation (Taylor et al. 2005), Notch1 cell signalling blockade (Carlen et al. 2009), and exposure to growth factors (Gregg and Weiss 2003). In addition, a hallmark of all stem cells is the asymmetric cell division, a feature that has also been observed in ependymal cells after brain injury (Gleason et al. 2008). And finally, it has been demonstrated that ependymal cells can proliferate under certain conditions such as injury of the nervous system (Gleason et al. 2008; Meletis et al. 2008; Cizkova et al. 2009; Moreno-Manzano et al. 2009) and stroke (Carlen et al. 2009; Li et al. 2002; Zhang et al. 2005, 2007), rendering a progeny consisting of neurons (Carlen et al. 2009) or astrocytes and oligodendrocytes (Meletis et al. 2008). These features make ependymal cells a possible candidate for cell replacement or nervous tissue repair. Our group have developed a method to obtain pure primary cultures of ependymal cells from adult animals to study the factors that could trigger those changes. We used both FGF2 and EGF as they induce the development of neurospheres from NSC.

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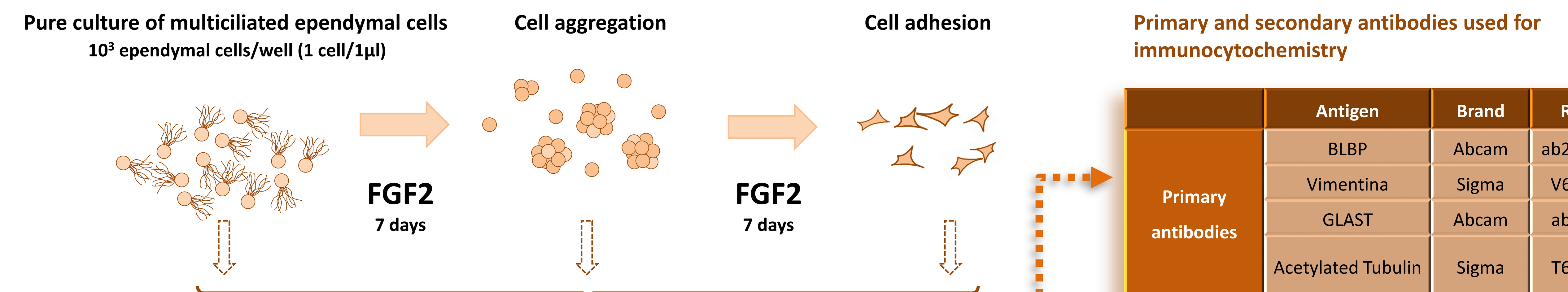
Material and methods

Pure cultures of multiciliated ependymal cells from adult rats were obtained by using the method published in Grondona et al. (2013). Once purified, ependymal cells do not attach to the culture plate and most of them rotate by the action of the cilia. The culture medium was alpha-MEM (Invitrogen) with 0.2% of Pluronic® F-127, 0.3% glucose, and 0.01 M HEPES. Three independent ependymal cell purifications were performed to calculate the relative abundance of aggregates in each experimental situation.

Experimental conditions		FGF2	Heparin	DMSO	FGFR inhibitor	Anti-FGF2
Control	- FGF2			1.1 µg/ml		
Experimental	+ FGF2	5 ng/ml	2 µg/ml	1.1 µg/ml		
FGFR inhibitor (PD166866)	- FGF2				5 mM	
	+ FGF2	5 ng/ml	2 µg/ml		5 mM	
Anti-FGF2 (blocking Ab)	- FGF2					2.5 µg/ml
	+ FGF2	5 ng/ml	2 µg/ml			2.5 µg/ml

Numbers indicate the final concentrations in the medium. FGFR inhibitor was diluted in DMSO. A blocking antibody against FGF2 (clone bFM-1, Millipore ref. 05-117) was used to block the function of FGF2.



Cells/aggregates from three stages were **fixed in formalin** and used for:

- **immunostaining** with glial radial markers
- in the case of the cell aggregation stage, **projected area quantification** was performed both in cells and aggregates

Projected area quantification of cells and aggregates was done in 96-well plates. At least 22 photographs were taken along two transects in each well. The ImageJ software was used for the determination of projected areas. Aggregates were classified into three categories according to its size: small (< 300 µm²), medium (300 –700 µm²) and large (> 700 µm²). Statistical analysis was performed with one-way ANOVA with Tukey Post Hoc (HSD) test to compare the effects of treatments.

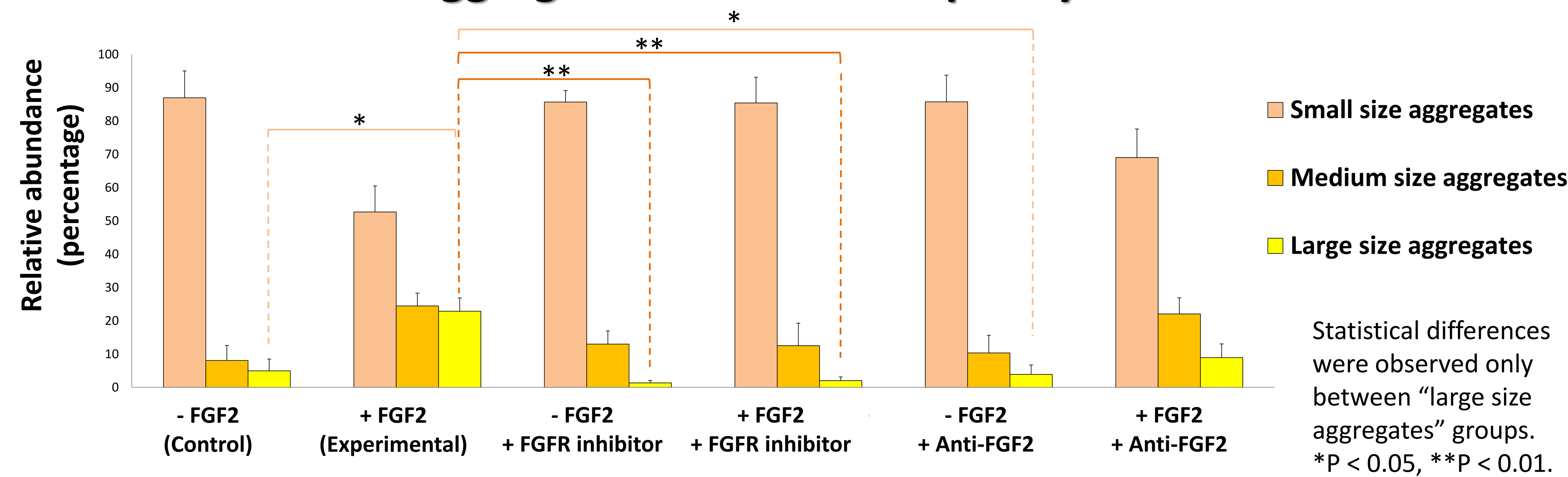
Primary and secondary antibodies used for immunocytochemistry

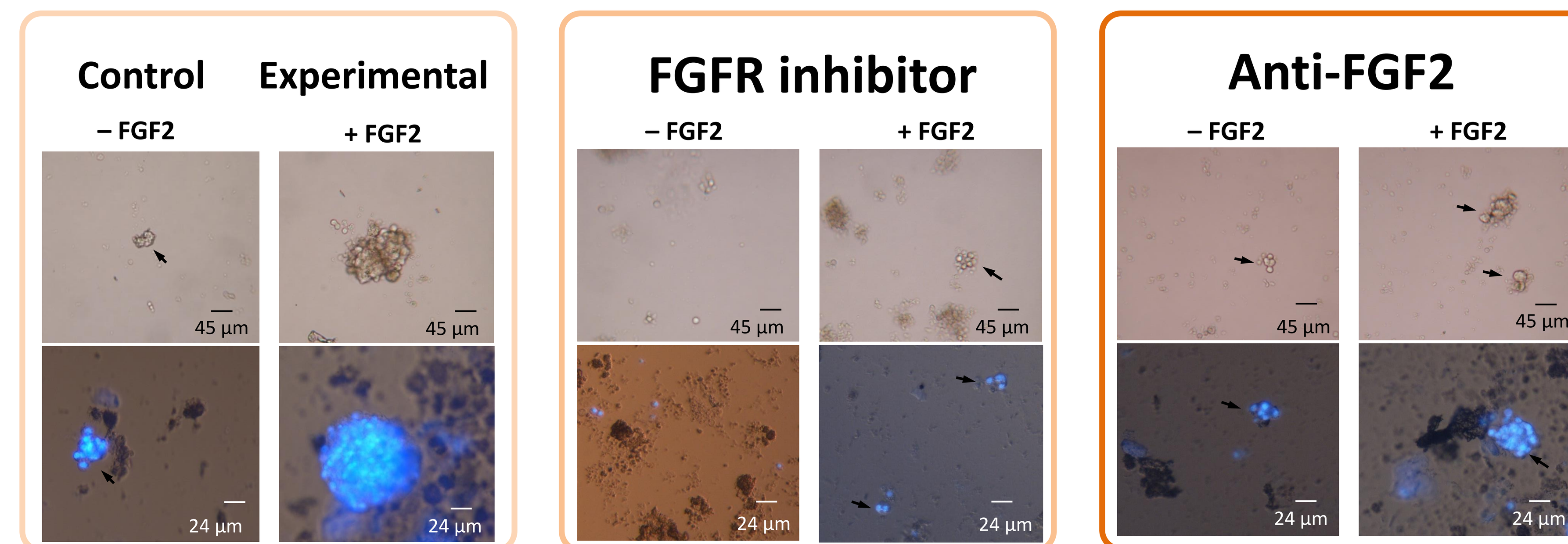
	Antigen	Brand	Ref.
Primary antibodies	BLBP	Abcam	ab27171
	Vimentina	Sigma	V6630
	GLAST	Abcam	ab416
	Acetylated Tubulin	Sigma	T6793

Antibodies were diluted 1:1000 in PBS pH 7.2 containing BSA (3%) and Triton X-100 (0.3%). Secondary antibodies were labelled with Alexa Fluor® 488 (rabbit IgG) and Cy3® (mouse IgG).

Results

FGF2 induces aggregation of isolated ependymal cells in vitro

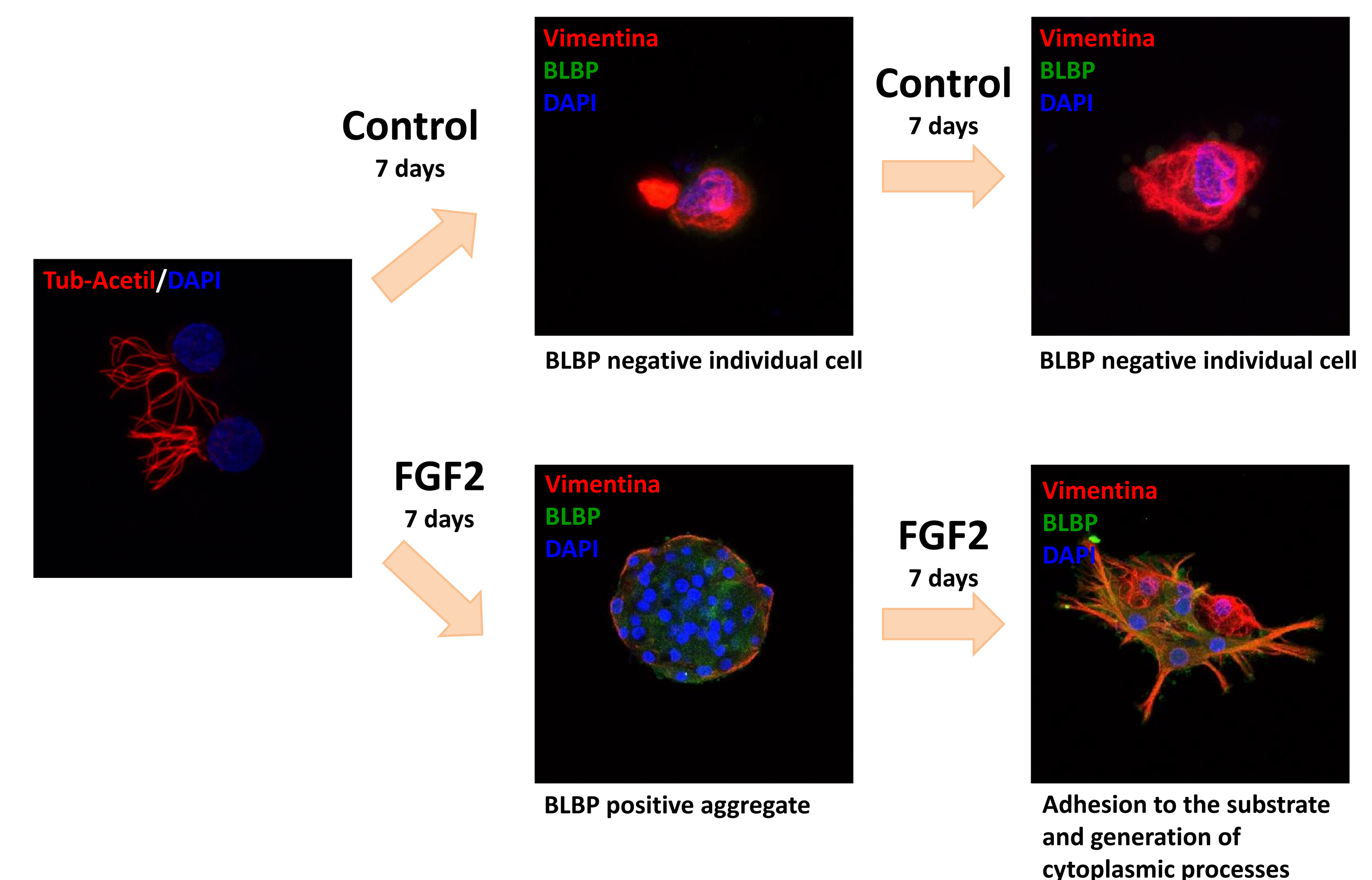




The effects of FGF2 on cell aggregation is specific:

1. It is abolished by an FGFR inhibitor.
2. It is blocked by a functional antibody against FGF2.

FGF2 induces ependymal cells to acquire radial glial features and astrocytic morphology



Conclusions

- The effect of both FGF2 and EGF on the differentiation status of ependymal cells was tested on pure cultures.
- FGF2, but not EGF, induces ependymal cells to aggregate into spheres.
- Cells in the spheres express radial glial markers (BLBP, GLAST and GFAP).
- Under the action of FGF2, cells from aggregates display cellular processes and adopt an astrocytic morphology.

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