LETTERS

Notch signalling regulates stem cell numbers *in vitro* and *in vivo*

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The hope of developing new transplantation therapies for degenerative diseases is limited by inefficient stem cell growth and immunological incompatibility with the host[1,2]. Here we show that Notch receptor activation induces the expression of the specific target genes hairy and enhancer of split 3 (*Hes3*) and Sonic hedgehog (*Shh*) through rapid activation of cytoplasmic signals, including the serine/threonine kinase Akt, the transcription factor STAT3 and mammalian target of rapamycin, and thereby promotes the survival of neural stem cells. In both murine somatic and human embryonic stem cells, these positive signals are opposed by a control mechanism that involves the p38 mitogen-activated protein kinase. Transient administration of Notch ligands to the brain of adult rats increases the numbers of newly generated precursor cells and improves motor skills after ischaemic injury. These data indicate that stem cell expansion *in vitro* and *in vivo*, two central goals of regenerative medicine, may be achieved by Notch ligands through a pathway that is fundamental to development and cancer[3–5].

New cell therapies based on embryonic stem (ES) cells are supported by work in animal models of human disease. They are difficult to implement, however, because it is hard to grow tissue-specific precursors in the laboratory and it is difficult to deliver them to diffuse disease sites in the body without stimulating an immune response. The results that we present here suggest a general model of stem cell expansion that applies to many precursor cells of clinical interest and that may lead to strategies that promote regenerative responses through activation of endogenous cells (Supplementary Fig. 1).

Notch encodes a transmembrane receptor that is cleaved to release an intracellular domain (Nicd) that is directly involved in transcriptional control[3,6]. Many components of the Notch pathway are expressed in the precursor cell compartment of the developing vertebrate central nervous system (CNS)[7,8]. Here, cell culture conditions that support the growth of a homogeneous population of fetal neural stem cells (NSCs) *in vitro* were used to define the action of Notch ligands, Delta-like 4 (Dll4) and Jagged 1 (Jag1)[9]. Reaction with an antibody specific for the cleaved cytoplasmic fragment showed proteolysis of the transmembrane form of the Notch receptor (Fig. 1a and Supplementary Fig. 2a). When cells were continuously observed in a sealed chamber on a microscope stage, Dll4 rapidly reduced cell death (Fig. 1b and Supplementary Fig. 2b). This survival effect of Notch ligands was antagonized by DAPT, a γ-secretase inhibitor that blocks Notch cleavage by Presenilin 1 (Fig. 2a). Cells exposed to Notch ligands retained the potential to generate neurons, astrocytes and oligodendrocytes after prolonged exposure to Notch ligands (Supplementary Table 1). These data show that activation of the Notch receptor rapidly inhibits cell death in NSCs.

The rapid effect of Dll4 on NSC survival suggested that cytoplasmic survival signals were induced in addition to slower transcriptional responses traditionally attributed to Notch activation. The insulin receptor stimulates cell survival through a cytoplasmic phosphorylation cascade that is initiated by phosphatidylinositol-3-OH kinase (PI(3)K) and the serine/threonine kinase Akt[4,10,11]. Dll4 induced DAPT-dependent phosphorylation of two principal activation sites on Akt with a peak at 5 min (Fig. 1c, d). Downstream of Akt, mammalian target of rapamycin (mTOR) is a key regulator of cell growth[12]. Jag1 caused transient phosphorylation of mTOR (Supplementary Fig. 2c). Like DAPT, the mTOR inhibitor rapamycin blocked the survival effect of Dll4 (Fig. 2a). These results suggest that Notch cleavage activates the survival cascade downstream of the insulin receptor.

STAT3 is a transcription factor activated at the cell surface by the gp130/LIF receptor and the JAK tyrosine kinase[5]. Extracellular ligands that promote phosphorylation of Tyr 705 on STAT3 stimulate the differentiation of CNS stem cells to a glia fate[13]. In our work on the role of STAT3 in fate choice, we noted that phosphorylation of a serine at position 727 correlated with the survival of NSCs. Dll4 and Jag1 induced dose-dependent phosphorylation of STAT3 on Ser 727 in the absence of Tyr 705 phosphorylation. Phosphorylation of Ser 727 peaked at 20 min, consistent with the kinetics of mTOR activation (Fig. 1e and Supplementary Fig. 2d). Ser 727 phosphorylation after Notch activation was sensitive to DAPT and rapamycin (Supplementary Fig. 2e). DAPT also reduced the basal amounts of phosphorylated Ser 727, suggesting that endogenous γ-secretase has a role in Ser 727 phosphorylation. These results suggest that serine phosphorylation of STAT3 mediates the survival effects of Notch activation.

We used a genetic approach to assess further the role of Ser 727 in cell survival pathways. NSCs were transfected with wild-type STAT3 and two mutant forms in which Tyr 705 or Ser 727 was altered to a 'neutral' amino acid (STAT3-YF and STAT3-SA). A gene encoding green fluorescent protein (GFP) in the plasmids enabled the number of transfected cells to be measured at 24 h and 4 d. The initial transfection efficiency was similar with all three complementary DNAs (~50% GFP^+ cells at 24 h). At 4 d, there were similar numbers of wild-type and STAT3-YF transfected cells (Fig. 1f; wild-type STAT3, 100 ± 29%; STAT3-YF, 100 ± 27%) but the proportion of STAT3-SA transfected cells was greatly reduced (16 ± 11%). This result suggests that endogenous ligands stimulate phosphorylation of STAT3 on Ser 727. Similar results were obtained in the presence of Dll4 (wild-type STAT3, 100 ± 29%; STAT3-YF, 109 ± 23%; STAT3-SA, 20 ± 3%). These data show that Notch ligands achieve their survival effects only when Ser 727 of STAT3 can be phosphorylated.

Phosphorylation on Tyr 705 is thought to control transcription of the gene encoding STAT3 and also mediates cell survival in cancer cells[5,14]. A form of STAT3 modified to prevent tyrosine

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phosphorylation and to have constitutively active serine phosphorylation was used to confirm a specific survival role for this target of the Notch receptor pathway. Transfection with a plasmid encoding a Ser 727 phosphomimetic and Tyr705 → Phe double mutant (STAT3-YF/SE) significantly increased colony formation (Fig. 1f; STAT3-YF 100 ± 20%, STAT3-YF or STAT3-SE 305 ± 58%), suggesting that Ser 727 has a positive role in NSC survival.

The short duration of mTOR activation suggests the presence of a negative regulatory mechanism that limits the extent of the positive signal. The PDK1 and p70 ribosomal S6 kinase components of the insulin signalling pathway are known to limit mTOR activation through the MSK1 and LKB1 kinases, which have been intensively studied as drug targets in diabetes and cancer[15,16]. Jag1 induced phosphorylation of MSK1 and LKB1 at 1 h after treatment, consistent with the presence of an inhibitory mechanism (Supplementary Fig. 2f).

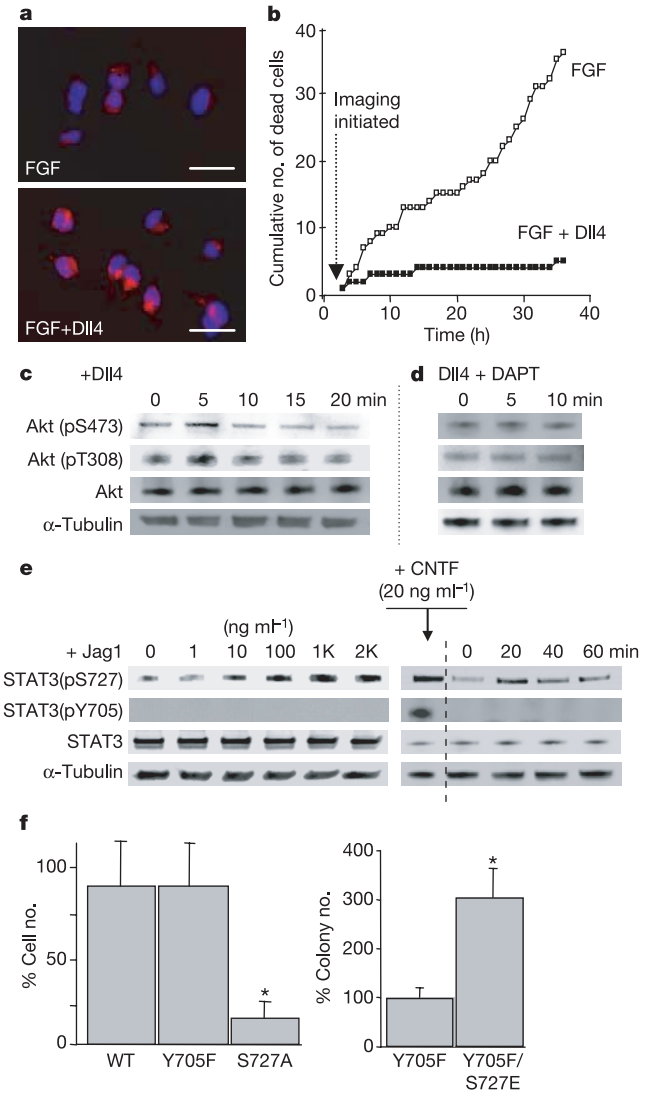

Figure 1 | Notch ligands activate second messenger signalling and support NSC (E13.5) survival *in vitro*. **a**, Dll4 (500 ng ml^{-1}, 1-h treatment) activates Notch1 (red, cleaved Notch1; blue, DAPI; scale bars 50 μm). **b**, Dll4 inhibits cell death in real-time cell lineage experiments. **c**, **d**, Dll4 activates Akt, with a peak at 5 min, in a DAPT-dependent manner. **e**, Jag1 induces phosphorylation of STAT3 on Ser 727 in a dose- and time-dependent manner (CNTF, positive control). **f**, Transfection with STAT3-YF does not alter survival relative to wild-type STAT3, whereas STAT3-SA inhibits growth and STAT3-YF/SE promotes survival (mean ± s.d. data 4 d after transfection).

The p38 mitogen-activated protein kinase is also a potential inhibitor of survival because it acts downstream of JAK and antagonizes growth in many cell types by activating MSK[17,18]. JAK and p38 inhibitors increased survival in NSCs (Fig. 2a). Combined JAK and p38 inhibition did not substantially improve survival, further indicating that JAK may act through p38 to antagonize the survival pathway in NSC. These data suggest that Notch acting through STAT3 promotes, and that p38 antagonizes, survival.

These results raise the issue of whether the cytoplasmic functions of the Notch receptor regulate transcription of the *Hes*/*Hey* gene family. Jag1 induced rapid transcriptional activation of *Hes3*, a gene with roles in NSC maintenance *in vivo*[8]. The regulation of *Hes3* messenger RNA by Jag1 was blocked when JAK kinase was activated by ciliary neurotrophic factor (CNTF) (20 ng ml^{-1}); this block was overcome by a JAK inhibitor. Induction of *Hes3* mRNA was also blocked by DAPT and rapamycin (Fig. 2b). *Hes3* mRNA and Hes3 protein are both enriched in cells of the adult subventricular zone (SVZ), a restricted site in the adult CNS that sustains stem cells[19] (Fig. 2c). Sonic hedgehog (Shh) is a secreted protein with mitogenic functions in precursor cells in the developing brain and a is chief target for treatments of paediatric brain tumours[20,21]. Jag1 induced long-term expression of Shh protein (Fig. 2d); cDNA transfection demonstrated that this effect is downstream of Hes3 (Fig. 2e). Previous studies show that Notch promotes NSC survival and that Nicd interacts with JAK and STAT[22,23]. The data presented here suggest a model in which Notch ligands act through a series of rapid and precisely timed cytoplasmic responses that have been widely studied in the context of cancer (Fig. 3).

It is important to establish whether this control mechanism applies

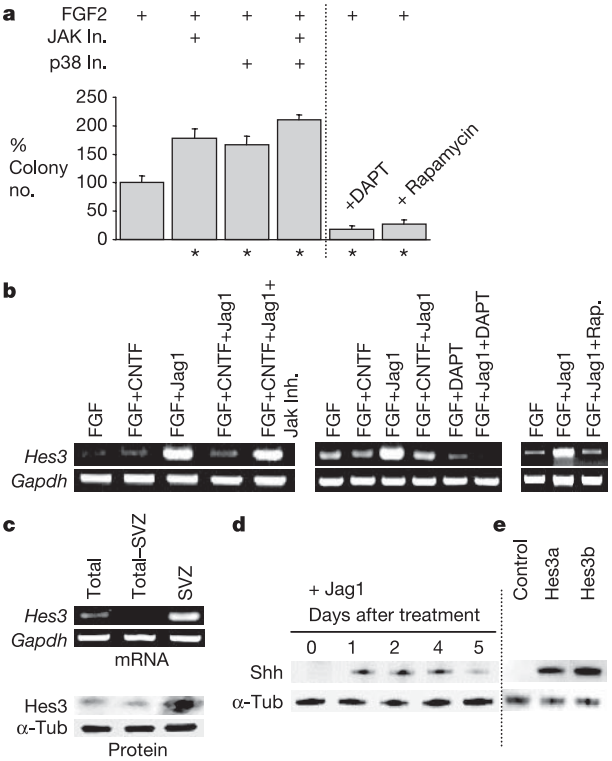

Figure 2 | Mediators and modulators of Notch signalling in NSC (E13.5) cultures. **a**, Inhibitors of JAK and p38 promote CNS stem cell survival; inhibitors of γ-secretase and mTOR inhibit survival (mean ± s.d. data 5 d after plating). **b**, CNTF (20 ng ml^{-1}) inhibits the Jag1-induced upregulation of *Hes3* mRNA; this effect is sensitive to a JAK inhibitor (200 nM). *Hes3* regulation by Jag1 is also rapamycin sensitive. **c**, *Hes3* mRNA and Hes3 protein are concentrated in the SVZ of adult mouse brains. **d**, Jag1 induces Shh expression in a time-dependent manner. **e**, Transfection with Hes3a or Hes3b induces Shh expression.

generally to ES and somatic stem cells. The clinical value of human ES (hES) cells is widely discussed but these cells are difficult to maintain in culture. Medium conditioned by mouse embryonic fibroblasts (MEFs) promotes their expansion. Although Akt and STAT3 have important roles in mouse ES cells, a clear function for STAT3 has not been defined in hES cells[24,25]. Phosphorylation of STAT3 on Tyr 705 was undetectable in hES cells, but MEF conditioned medium maintained Ser 727 phosphorylation that was insensitive to JAK inhibition (Fig. 4a). High concentrations of fibroblast growth factor 2 (FGF2) and bone morphogenetic protein inhibitors are currently used to support human ES cell growth[2,26]. Notch activation and inhibition of JAK and p38 (daily for 7 d) increased colony formation without affecting colony size in HSF6 cells (Fig. 4b). DAPT reduced colony number (control, 100 ± 5%; DAPT 51 ± 11%). Inhibition of JAK also increased survival in H1 and H9 hES cells (Supplementary Table 2).

To assess whether JAK inhibition was consistent with longer-term maintenance of the undifferentiated state, HSF6 cells were continuously exposed to the JAK inhibitor by daily additions for three passages (3 weeks). The cells had a normal morphology and antigen profile ($Oct3/4^+$/$SSEA4^+$/$Tra\text{-}1\text{-}60^+$/$Tra\text{-}1\text{-}81^+$/$SSEA1^-$), and retained their ability to generate $Sox1^+$/$Nestin^+$ neural precursors and tyrosine-hydroxylase-positive neurons (data not shown). Similar results were obtained with endocrine precursors when cells from the developing pancreas were placed in culture (Fig. 4c, d). These data suggest that common regulators control the *in vitro* expansion of pluripotent and somatic stem cells. These cytoplasmic responses to Notch receptor activation may also contribute to the developmental inhibitory functions of Notch defined by genetic experiments in *Drosophila*[6].

To determine whether Notch ligands are active *in vivo*, osmotic pumps were used to administer artificial cerebrospinal fluid (ACSF), FGF2, Dll4, or a combination of FGF2 plus Dll4 (Dll4, 4.2 μg ml^{-1}; FGF2, 2.5 μg ml^{-1}; $n = 6$ per treatment) for 7 d to the normal adult brain. Twice-daily injections of 5-bromodeoxyuridine (BrdU) from days 2 to 6 were given to label dividing cells. Dll4 increased the number of BrdU-positive cells in both the ispi- and contralateral SVZ in a dose-dependent manner (Supplementary Fig. 3a). By contrast, FGF2 was ineffective (Supplementary Fig. 3b). Many newly generated cells expressed doublecortin, a protein that identifies immature neurons[27] (Supplementary Fig. 3c). Cells generated during the days after Notch ligand delivery were found at 45 d in the cerebral cortex; these cells were largely GFAP-negative and rarely (<1%) expressed the neuronal markers NeuN and calretinin (data not shown); however, many BrdU-positive cells in the cerebral cortex expressed the early neuronal marker HU (ipsilateral, 13.95 ± 0.46%; contralateral, 10.2 ± 2.35%; Supplementary Fig. 3d). These data show that cells stimulated to divide by Dll4 survive for long periods in the parenchyma of the normal brain in an immature state. This result was seen in the absence of injury, indicating that Notch signalling may regulate the activity or size of the stem cell compartment in uncompromised adult tissues.

An injury model in which focal ischaemia was restricted to the cerebral cortex was used to determine whether Notch ligands promote regenerative responses. The middle cerebral artery was occluded at the surface of the brain to generate an injury restricted to the cerebral cortex. Exogenous proteins were delivered by osmotic pump, and standardized tests were used to measure motor behaviour over a 45-d survival period[28]. In this injury model, Dll4 and FGF2 increased the numbers of BrdU-positive cells in both the ipsi- and

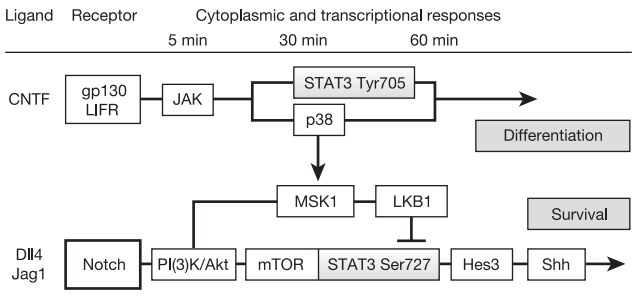

Figure 3 | A stem cell signalling pathway that affects survival and differentiation. Notch activation is followed by phosphorylation of Akt, mTOR and STAT3 on Ser 727, and by subsequent induction of Hes3 and Shh, in a temporally controlled order. Notch cleavage also simultaneously activates an opposing pathway that limits growth through MSK1 and LKB1. This opposing pathway can be accessed by gp130-dependent ligands that activate JAK and p38.

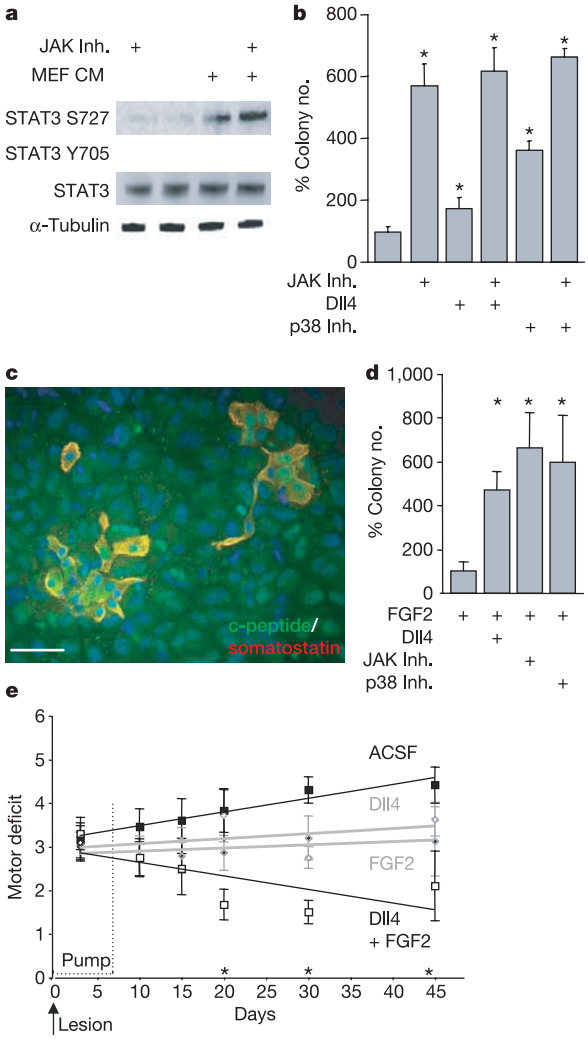

Figure 4 | *In vitro* and *in vivo* data support a general role for the signalling model in stem cell biology. **a**, MEF conditioned medium (MEF CM) stimulates phosphorylation of STAT3 on Ser 727: withdrawal of MEF CM (16 h) causes almost complete loss of Ser 727 phosphorylation without affecting STAT3 protein amounts in MEF-free hES cultures plated as aggregates; phosphorylation of STAT3 on Tyr 705 is undetectable in all conditions shown. **b**, Dll4, JAK inhibitor and p38 inhibitor increase plating efficiency of HSF6 hES cells plated as single dissociated cells (mean ± s.d. values 6 d after plating). **c**, Dll4 supports pancreatic endocrine precursor cultures expressing c-peptide and somatostatin (7 d after plating). **d**, Dll4, JAK inhibitor and p38 inhibitor promote survival of fetal pancreatic cultures (data are mean ± s.d. at 7 d). **e**, Treatment with Dll4 (42 μg ml^{-1} in pump) plus FGF2 (2.5 μg ml^{-1} in pump) improves motor skills of ischaemic rats (regression lines assume linear rate of change over the whole period of observation; data are mean ± s.e.m.).

contralateral SVZ (Supplementary Fig. 3e). Here again, very few of the BrdU-labelled cells expressed markers characteristic of differentiated neurons or astrocytes. In lesioned rats treated with ACSF, there was a progressive deterioration in motor performance (Fig. 4e). The groups treated with either FGF or Dll4 alone showed no change in their motor scores over 45 d. Rats treated with both FGF2 and Dll4 showed significant improvement in motor skills. The size of the lesion was comparable in all treatment groups (21.3 ± 2.5%, ACSF; 21.0 ± 3.5%, FGF2; 20.5 ± 2.9%, FGF2 + Dll4; % of hemisphere volume at 7 d). The lack of an immediate neuroprotective effect suggests the exogenous proteins do not have a strong short-term anti-inflammatory function[29]. Notch is important in all tissues and the beneficial effects of Notch ligands may involve responses in cells of the vascular, immune and nervous systems. *In vitro* data suggest that the Notch receptor has an important role in responses to low-oxygen conditions[30]. The powerful *in vivo* effect that we have shown here indicates that Notch ligands may stimulate regenerative responses to the oxygen deprivation that follows ischaemia *in vivo*. Further studies will define how interactions between Notch ligands and other growth factors can be optimized to obtain more complete differentiation and recovery.

METHODS

Full methods are given in the Supplementary Information.

Cell culture. CNS stem cells and pancreatic precursors were cultured from mouse embryos at embryo day 13.5 (E13.5). Human ES cell lines (HSF6, H1, H9) were maintained on MEFs according to the suppliers' protocols.

Reverse transcriptase PCR. Newly designed and previously published (a gift from R. Kageyama, Institute for Virus Research, Kyoto University, Japan) primers were used (see Supplementary Information).

Nucleofection. STAT3 (a gift from T. Kitamura, Institute of Medical Sciences, Tokyo, Japan) and Hes3 (a gift from R. Kageyama) plasmid DNAs were expressed in CNS stem cells using the VPG-1004 nucleofector kit (Amaxa).

***In vivo* experiments.** Drugs were administered over 7 d by an osmotic pump (Azlet). Rats received intraperitoneal injections of BrdU (50 mg per kg) every 12 h for 5 d beginning on day 1 after the operation. For the induction of focal ischaemia in rats, male spontaneously hypertensive rats underwent permanent middle cerebral artery occlusion[28].

Statistical analysis. Results shown are the mean ± s.d. or mean ± s.e.m. as indicated. Asterisks identify experimental groups that were significantly different from control groups by the Student's *t*-test (Microsoft Excel), with a Bonferroni correction for multiple comparisons (α-value, 0.05), where applicable.

Reagents. Reagents are listed in Supplementary Information.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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