Article is available online at http://www.webio.hu/por/2007/13/4/0302

ARTICLE

Transcriptional Characterization of the Notch Signaling Pathway in Rodent Multipotent Adult Progenitor Cells

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The Notch signaling pathway is a multifunctional, evolutionarily conserved pathway, which plays an important role in development as well as stem cell biology. Multipotent adult progenitor cells (MAPCs) represent a unique stem cell population, which is capable of differentiating into cell types of the ectodermal, mesodermal and endodermal lineages in vitro, and contribute to most somatic cell types in vivo. Our aim was to characterize the gene expression of Notch signaling elements in rodent MAPCs. We show that transcripts for Notch-receptors, ligands, regulatory molecules of the pathway and the Hairy/Enhancer of Split-1 (HES-1) target gene are present in mouse and rat low-Oct4 MAPCs. We found that mouse Notch3 and rat Notch1 transcripts increased when cells were cultured at high density for 48 to 96 hours. HES-1 and HES-related transcription factor-1 (HERP-1), transcriptional targets of Notch-signaling, were both elicited by immobilized Delta1 ligand. In addition, mRNA for Notch1 and Notch3 was also induced by Notch-signaling, suggesting the presence of regulatory feedback loops. Slight differences between mouse and rat derived MAPCs suggest that the exact function, transcriptional regulation and the fine-tuning of the signal may be species specific. Taken together, we characterized the gene expression profile of the Notch pathway in rodent low-Oct4-MAPCs, and showed that the pathway is functional and can be modulated. Our results provide an additional tool and a further basis for a better understanding of stem cell biology. (Pathology Oncology Research Vol 13, No 4, 302–310)

Key words: Notch, HES-1, multipotent adult progenitor cell, stem cell

Introduction

The Notch signaling pathway

Notch belongs to a family of evolutionarily conserved proteins that are involved in a number of cellular and developmental functions.[1] In mammals, four Notch receptors (Notch1-4) and five Notch ligands (Jagged1, Jagged2, Delta1, Delta3 and Delta4) have been identified. Upon ligand binding the receptor is proteolytically cleaved by a γ-secretase complex containing the Presenilin-1 or Presenilin-2 enzyme, and the intracellular domain of the receptor translocates into the nucleus of the receiving cell, where it binds to the CSL transcription factor (*C*BF1 in mammals, *S*uppressor of Hairless in *Drosophila* and *L*ag in *Caenorhabditis elegans*; also known as RBP-Jκ), and converts the originally inhibitory complex into a transcriptional activator.[2-4] The best characterized transcriptional targets belong to the Hairy/Enhancer of Split (HES) and the HES-related repressor protein (HERP; also known as HES-related transcription factor with YPRW motif, HRT or Hey)

Received: Oct 10, 2007; *accepted:* Dec 5, 2007

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This work was supported by the Forum Engelberg/Gertrud von Speyr Foundation (Switzerland) and the National Science Foundation of Hungary (TS049887).

families.[4] HES and HERP are basic helix-loop-helix transcription factors that negatively regulate the expression of downstream target genes, such as tissue specific transcription factors, regulating lineage-specific differentiation. HES and HERP proteins also repress transcription through the formation of homodimers and binding DNA directly – such direct target genes may be the Mash1 proneural transcription factor and the CD4 protein in T-cells. Indirect repression is also possible by binding and sequestering other transcription factors – for example the muscle-specific factor MyoD, or the coactivator p300 – and preventing their complex formation with transcriptional partners.[4,5]

Several molecules have been described which regulate Notch function. Deltex is an E3 ubiquitin ligase, which may cooperate with Notch both in an agonistic and antagonistic manner, and may participate in signaling pathways – at least in part – independently of Notch.[6] The Numb adapter protein promotes the ubiquitination and degradation of Notch1, thereby negatively regulating Notch function.[7,8] Signal transduction can be differentially modulated through receptor glycosylation by the family of the Fringe glycosyl-transferases (Lunatic, Manic and Radical Fringe), attenuating Jagged-mediated signaling and potentiating Delta-mediated signaling.[9]

The importance of Notch signaling in stem cells and cell fate specification

Throughout development, Notch-signaling regulates cell fate determination by inhibiting differentiation along default pathways, and allowing cells to adopt alternative cells fates when proper signals are delivered.[10] This phenomenon is called lateral inhibition. However, Notch can also instructively promote cell differentiation, such as during glial development.[11] The diverse roles of Notch in stem cell systems and in embryonic development are highlighted by a number of studies involving genetically engineered animals and *in vitro* approaches.[12]

Several stem cell types were shown to express elements of the Notch pathway.[12-14] Notch1, 2 and 3 receptors, Delta1 and HES-1 were identified in embryonic stem cells (ESCs) on the mRNA or protein level.[14-16] Many components of the Notch pathway are also expressed in the precursor cells of the developing vertebrate central nervous system,[17] and the activity of the pathway is required for the maintenance, but not the generation, of mouse neural stem cells, and it promotes survival of neural stem cells *in vitro* and *in vivo*.[18,19] Cancer stem cells share several properties with normal, non-neoplastic stem cells, and they have been implicated to utilize similar signaling pathways. Interestingly, the blockade of Notch signaling results in the depletion of stem-like cells and suppresses tumor formation in medulloblastomas, which are thought to arise from primitive neural stem or precursor cells.[20] The delicate balance between stem cell self-renewal and differentiation has to be tightly controlled by a network of signaling routes, in which Notch cross-talks with several other pathways, such as the Wnt,[21] the PKB/Akt/mTOR[22] and the JAK/STAT pathway.[18,23]

The Notch pathway is also active in mouse hematopoetic stem cells and its function is necessary to maintain an undifferentiated state and to prevent lineage commitment.[21] Immortalized, pluripotent, cytokine-dependent hematopoetic cell lines can be established by transfection with a constitutively active Notch1 construct.[24] Ligand-activated Notch enhances the proliferation of hematopoetic stem and progenitor cells, and regulates their differentiation potential.[25,26] However, inhibitory effects of the ligands have also been published,[27] which suggests that responsiveness to Notch-signals is likely to be cell type and context dependent.[13] The role of Notch-signaling has been demonstrated in endothelial arteriovenous specification as well.[28]

Multipotent adult progenitor cells

Stem cells have gained tremendous scientific interest in the last decade as they hold great potential for the treatment of degenerative or inherited diseases. Embryonic stem cells are pluripotent and can differentiate to virtually all cell lineages; however, undifferentiated embryonic stem cells are prone to teratoma formation when injected *in vivo*, and the creation of human embryonic stem cells remains ethically controversial.[29] Tissue specific adult stem cells were originally thought to have more restricted potential, but research in recent years has shown that they may still be capable of differentiating into cells other than the tissue of origin.[30] However, this remarkable plasticity is currently difficult to exploit in a therapeutical setting. Multipotent adult progenitor cells may represent a new, clinically useful and ethically acceptable source of pluripotent stem cells.

Multipotent adult progenitor cells (MAPCs) have been isolated from the adherent fraction of mouse, rat and human bone marrow cells, as well as murine muscle and brain.[31-33] MAPCs can differentiate into several ectodermal, mesodermal and endodermal cell types in vitro,[34,35] and transgenes from at least some of the lines have been demonstrated in almost all cell types of the three germ layers after injection of MAPCs into a mouse blastocyst. They can be maintained in culture for more than 120 population doublings with a normal karyotype. Of note, cell lines with different expression levels of the transcription factor POU5F1 (Oct4) are isolated from murine and rat bone marrow when cultured under MAPC conditions.[36,37] Cells with higher expression levels of POU5F1 (ΔCT levels compared with the housekeeping gene GAPDH between 2 and 8; high-Oct4 MAPCs) appear to differentiate more robustly to endothelial and endodermal cell types[37] compared with cells expressing lower levels of POU5F1 (ΔCT levels compared with the housekeeping gene GAPDH >8; low-Oct4 MAPCs). It

remains unknown whether the phenotype of MAPCs is culture induced or exists as such in vivo. However, Anjos-Afonso et al, terming the cells pre-MSC, have isolated cells from mouse bone marrow based on SSEA1 expression, a cell surface determinant also present on ESCs.[38] SSEA1+ cells express Oct4 and SSEA1- cells do not express Oct4 upon isolation from bone marrow. Furthermore, SSEA1- cells cannot be induced to express Oct4 in culture, suggesting that if MAPCs are created in vitro, they can only be created from a subpopulation of tissue cells.

The routine use of MAPCs has been hampered by the requirement of tedious culture conditions and very low cell density,[36] which is crucial for maintaining the stem cell phenotype; high cell density gradually leads to the loss of stem cell properties, more so in populations of cells that express lower levels of POU5F1 (Park Y et al, manuscript in preparation). The reason for this is not known, but soluble factors as well as cell contact dependent interactions may play an important role. Preliminary gene expression array data showed that the expression of Notch3 and HES-1 increased when MAPCs expressing low levels of POU5F1 were allowed to proliferate to confluence (Lenvik T, Verfaillie CM, unpublished observations). Given the importance of Notch in cell-cell interactions, and its role in stem cell maintenance and differentiation, it is likely to contribute to the biological properties of MAPCs as well.

In the present work we characterized the gene expression of Notch pathway elements in rodent MAPCs expressing Oct4 albeit at low level, which provides a functional basis for the modulation of the signal. In addition, several components of the pathway were identified, which were themselves target genes of Notch signaling.

Materials and Methods

Cell isolation and culture

Mouse and rat multipotent adult progenitor cells (MAPCs) were isolated from the bone marrow, brain and skeletal muscle of 3-5 week old ROSA26 mice and from the bone marrow of Sprague-Dawley rats and cultured with a modification of a previously described procedure.[33] All protocols were approved by the University of Minnesota Institutional Animal Care and Use Committee. Briefly, cells were seeded on plates coated with 10 ng/ml (for rat) or 100 ng/ml (for mouse) fibronectin (FN; Sigma, St. Louis, MO) in expansion medium at $6x10^5$ cells/cm^2 and cultured at high density for 3-4 weeks. After 3-4 weeks, CD45$^+$/Ter119$^+$ cells were depleted using micromagnetic beads (Miltenyi Biotec, Auburn, CA), replated at 10 cells/well in FN coated 96-well plates and expanded at densities between 100 and 1000 cells/cm^2.

MAPC expansion medium consisted of 60% DMEM-low glucose (Gibco BRL, Carlsbad, CA) and 40% MCDB-201 (Sigma) supplemented with 1X insulin-transferrin-selenium (ITS) (Sigma), 1X linoleic acid-bovine serum albumin (LA-BSA) (Sigma), 10^{-9} M dexamethasone (Sigma), 10^{-4} M ascorbic acid 2-phosphate (Sigma), 100 units of penicillin/1000 units of streptomycin (Cellgro, Herndon, VA), 2% fetal bovine serum (FBS; Hyclone, Logan, UT), 10ng/ml hPDGF-BB (R&D Systems, Minneapolis, MN), 10 ng/ml mEGF (Sigma) and 1000 units/ml mLIF (ESGRO, Temecula, CA). In certain mouse cell cultures dexamethasone was omitted, ITS was replaced by 1X SITE supplement (Sigma), LA-BSA was reduced to 0.2X, and Chemically Defined Lipid Concentrate (Gibco), 0.8 mg/ml BSA (Sigma) and 55 μM β-mercaptoethanol (β-ME, Gibco) was added. Mouse cells were grown on plates (Nalge Nunc International, Rochester, NY) coated with 100 ng/ml FN in 5% CO_2 and 5% O_2 in a humidified chamber. Rat cells were grown in flasks (Corning, Corning, NY) coated with 10 ng/ml FN in 5% CO_2 and 20% O_2 in a humidified chamber. Cell density (200-1000 cells/cm^2) was maintained by detaching cells with 0.25% trypsin (Cellgro) and replating every 36-48 hours. Photographs were taken on a Zeiss microscope with a Nikon Coolpix digital camera.

Cells were cultured at high density (>$2x10^4$ cells/cm^2) to assess density-dependent changes. Tri-lineage differentiation potential of MAPCs was determined as described previously.[33]

The R1 mouse embryonic stem cell line was a kind gift from Dr. Janet Rossant (Mount Sinai Hospital, Toronto, Canada).

Cell cultures with Delta-1$^{ext\text{-}IgG}$

Delta-1$^{ext\text{-}IgG}$ – a ligand of the Notch receptor – was kindly provided by Irwin D. Bernstein (Fred Hutchinson Cancer Research Center, Seattle, WA, USA). Four-well plastic chamber slides (Nalge Nunc) or 24-well plates (Corning) were coated overnight at 4°C with 10 μg/ml Delta-1$^{ext\text{-}IgG}$ in 10 ng/ml FN. Control wells were coated with 10 μg/ml hIgG (Sigma) in 10ng/ml FN. (A second control was also included with cells grown on FN only – values from the two types of control were not different from each other.) Mouse or rat MAPCs were seeded at $5x10^4$ cells/well in complete MAPC expansion medium (= medium with growth factors) or in medium without PDGF-BB, EGF, LIF and β-ME (= medium without growth factors), and cultured in a humidified chamber. CO_2 and O_2 concentrations corresponded to MAPC culture conditions as per cell type. Cells were lysed for RNA after 48 or 96 hours.

RNA isolation and quantitative real-time PCR

RNA was extracted with the RNeasy Mini kit (Qiagen, Valencia, CA) and treated with DNAse (DNA-free™; Ambion, Austin, TX). Reverse transcription was per-

Table 1. **Primers used for qRT-PCR**

Target gene	*Forward primer*	*Reverse primer*
Mouse and rat Notch1	TGGAGCTACCTGCACTGACTATC	TCTCCTCGGAGCAGTTAGACC
Mouse Notch2	CAAAGAAAGCCAAGGCTGAG	GTTCTGCCTGAGGAGGAGTG
Rat Notch2	CCTGAACGGGCAGTACATTT	GCGTAGCCCTTCAGACACTC
Mouse and rat Notch3	ATACACTGGCCCCTTCTGTG	GTCGAGGCAAGAACAGGAAA
Mouse Notch4	TGAATCGGAGGTTCTGGATG	CCACAGAAGACGGCTGACA
Mouse Jagged1	AAAGACCACTGCCGTACCAC	GGGGACCACAGACGTTAGAA
Mouse Jagged2	CTGCCATGAAAACATTGACG	CGTTGGGATTGATGTCACAG
Mouse Delta1	CTGTGACAAACCAGGGGAGT	GACAACCTGGGTATCGGATG
Mosue Delta4	CAGAGACTTCGCCAGGAAAC	TCATTTTGCTCGTCTGTTCG
Mouse HES-1	ACACCGGACAAACCAAAGAC	ATGCCGGGAGCTATCTTTCT
Rat HES-1	AAAGATAGCTCCCGGCATTC	CCTCACACGTGGACAGGAA
Mouse HERP1	GCGTCGGGATCGAATAAATA	AGCATGGGCATCAAAGTAGC
Mouse CBF1	CTCCACCCAAACGACTCACTA	GAAGAATGAGCACTGTTTGATCC
Mouse Deltex1	CTCCCCGTGAAGAACTTGAA	CACGTCGCTTTTGCTTACTG
Mouse Numb	CGGGAAAGAAAGCAGTGAAG	AGTGGTGCCATCACGACATA
Mouse Presenilin-1	AAGTACCTCCCCGAATGGAC	TCAGCCATATTCACCAACCA
Mouse Presenilin-2	CGTGTCGTGTTTACTTCGTGA	CTGGTGACAAGACAGGTAGCAC
Mouse Lunatic Fringe	CTGCAAGATGGCTGTGGAGTA	CGGAGGTTGACGTAGTTGTCAT
Mouse Manic Fringe	CGTGGTCACCAACTGTTCTG	GCTTGCCCACATAGACATCA
Mouse Radical Fringe	CTGCCGTCTGGTGCTATTTT	AAAGCTCCCTCAACCCTGTT
Mouse Oct4 (POU5F1)	CCAATCAGCTTGGGCTAGAG	CCTGGGAAAGGTGTCCTGTA
Rat Oct4 (POU5F1)	CTGTAACCGGCGCCAGAA	TGCATGGGAGAGCCCAGA
Mouse GAPDH	CATGGCCTTCCGTGTTCCTA	CTGGTCCTCAGTGTAGCCCAA

formed with the SuperScript™ First Strand Synthesis kit (Invitrogen, Carslbad, CA). Twenty-five ng cDNA underwent 40 cycles of amplification (ABI PRISM 7700; Perkin Elmer, Applied Biosystems, Foster City, CA) with a two step PCR reaction (95ºC for 15 seconds, 60°C for 60 seconds) after initial denaturation (95ºC for 10 minutes) with 100nM of each primer and 1X SYBR@ Green PCR Master Mix (Applied Biosystems). Primers used are listed in *Table 1*. Gene expression was normalized to the GAPDH housekeeping gene. Rat GAPDH primers were purchased from Applied Biosystems (TaqMan@ Rodent GAPDH Control Reagents).

Statistics

Paired Student's t-test was applied to evaluate significance; $p<0.05$ was considered statistically significant. Statistical analysis was performed using Microsoft Excel.

Results

Characterization of mouse and rat low-Oct4 MAPC

As we have recently described, isolation of clonal populations of cells under MAPC conditions yields populations of cells expressing variable levels of the ESC transcription factor Oct4 (POU5F1).[36,37] The cells used in the current study expressed relatively low levels of Oct4: for the murine cells used, ΔCT values of Oct4 compared with GAPDH were between 15 and 18; for rat cells the ΔCT values were between 11 and 15.

Notch-pathway profile in mouse MAPCs

The presence of Notch pathway transcripts was determined by real-time PCR in bone marrow derived mouse MAPCs; in addition, brain and muscle derived lines were included in some cases.

We found that transcripts for elements functioning at distinct levels of the signaling cascade were present in mouse MAPCs. Notch1, 2 and 3 receptors, the Jagged1 ligand, HES-1, Presenilin-1 and -2, CBF-1, Lunatic Fringe and Radical Fringe, and Numb transcripts were abundant in mouse MAPCs. Jagged2, Delta1, Notch4, Delta4, HERP-1, Deltex and Manic Fringe were expressed at low to undetectable levels.

When mouse MAPCs were compared to mouse ESCs, we found that Notch1 expression was similar in the two cell types; Notch2 expression was lower (about 6-fold), Notch3 expression was substantially higher (about 32-fold) and HES-1 expression was slightly higher (two to threefold) in mouse ESCs. Notch4, Jagged1 and 2, Delta1, Presenilins, CBF-1, Lunatic Fringe, Manic Fringe, Radical Fringe and Numb were readily detectable; HERP-1 and Deltex were moderately expressed in mouse ES cells. Delta4 was absent in mouse ESCs as well.

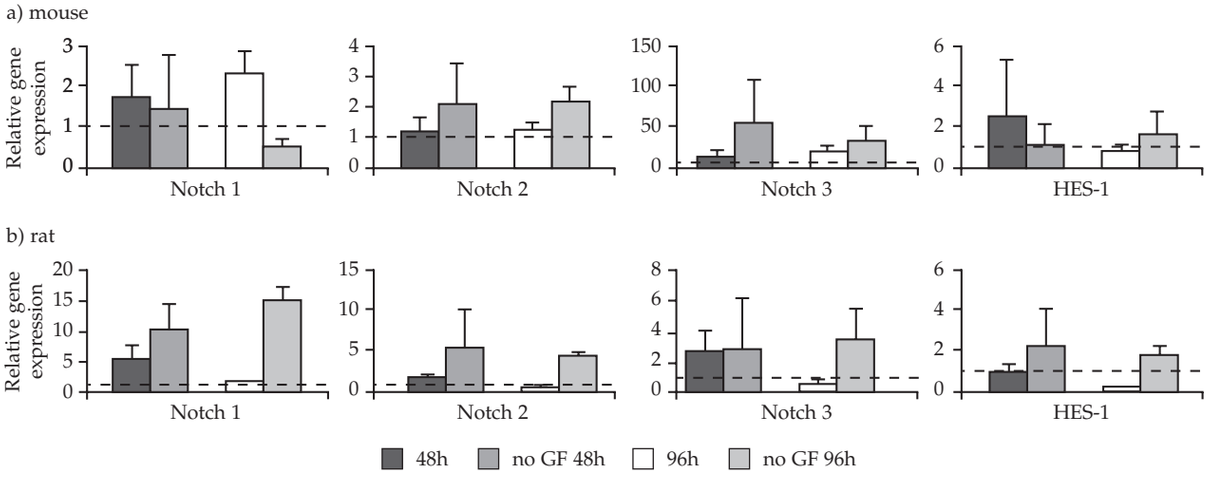

Figure 1. *Cell density dependent gene expression in* ***(a)*** *mouse and* ***(b)*** *rat low-Oct4-MAPCs. Relative gene expression of cells cultured at high cell density compared to cells cultured at low (MAPC) density (=1, indicated as a dashed line). Values are normalized to GAPDH. (GF: medium with EGF, PDGF and LIF; no GF: medium without EGF, PDGF and LIF; means and SD are shown for two to three independent experiments.)*

High cell density differentially regulates gene expression of Notch pathway elements

To assess short-term transcriptional changes induced by high cell density, mouse and rat MAPCs were cultured at $5x10^4$ cells/well in 4-well chamber slides in medium with or without EGF, PDGF and LIF, and gene expression was evaluated after 48 and 96 hours. Expression levels of Notch1, Notch2 and HES-1 in mouse MAPCs were not significantly altered upon maintenance at high cell density. However, Notch3 was significantly up-regulated in cultures with high cell density, which could still be observed after 96 hours (*Fig. 1a*); HERP-1 was also up-regulated at 48 hours, but its levels were still low and fell back to almost control values after 96 hours (not shown).

High cell density also influenced Notch-receptor expression in rat MAPCs (*Fig. 1b*): Notch-1 transcripts increased at 48 hours in medium with growth factors, and both at 48 and 96 hours in medium without growth factors. Notch-2 and Notch-3 expression was increased, albeit to a lesser extent. Changes in HES-1 expression were moderate. Taken together, mRNA expression of Notch-pathway components were differentially regulated by cell density in both murine and rat MAPCs.

Elements of the Notch pathway are target genes of Delta1 signaling in mouse and rat MAPCs

To test whether the Notch ligand Delta1 can elicit signaling through Notch receptors, MAPCs were cultured on plate-bound Delta-$1^{ext\text{-}IgG}$ in MAPC medium with or without growth factors. Delta1 treatment significantly increased HES-1 mRNA expression in mouse MAPCs, both in the presence and the absence of growth factors; this increase was sustained, and still detectable after 96 hours of culture. HERP-1 was dramatically up-regulated at all time points tested. Delta1 also up-regulated Notch1 and Notch3, whereas Notch2 levels did not show significant changes (*Table 2*).

A similar effect was observed in rat MAPCs: the presence of the ligand significantly increased Notch-1, Notch-3 and HES-1 expression in all conditions tested. Notch2 mRNA was also up-regulated in medium with growth fac-

Table 2. **Average fold expression changes of Notch pathway genes in mouse low-Oct4-MAPCs grown on Delta-$1^{ext\text{-}IgG}$ in complete MAPC medium or in medium without growth factors (no GF).**

	48h	*no GF 48h*	*96h*	*no GF 96h*
Notch1	4.88 (±1.73)x	5.40 (±1.67)x	2.2 (±0.81)x	8.2 (±2.26)x
Notch2	0.7 (±0.35)x	0.8 (±0.11)x	0.6 (±0.12)x	0.6 (±0.22)x
Notch3	46.6 (±22.38)x	48.3 (±2.25)x	4.8 (±1.03)x	53.3 (±17.05)x
HES-1	6.1 (±2.94)x	10.4 (±6.01)x	8.5 (±3.46)x	9.1 (±3.19)x
HERP-1	1030 (±989.65)x	99.1 (±2.91)x	27.3 (±7.15)x	1192.9 (±358.28)x

Values are relative to control cells (=1), normalized to GAPDH. (Means of two to three independent experiments ±SD are shown.)

Table 3. **Average fold expression changes of Notch pathway genes in rat low-Oct4-MAPCs grown on Delta-1$^{ext\text{-}IgG}$ in complete MAPC medium or in medium without growth factors (no GF).**

	48h	*no GF 48h*	*96h*	*no GF 96h*
Notch1	26.10 (±9.83)x	24.68 (±0.48)x	31.47 (±4.83)x	12.64 (±4.60)x
Notch2	1.14 (±0.46)x	0.75 (±0.22)x	3.53 (±1.27)x	0.60 (±0.05)x
Notch3	88.15 (±57.71)x	172.50 (±66.42)x	259.54 (±137.88)x	216.92 (±60.31)x
HES-1	4.83 (±2.34)x	3.86 (±0.36)x	5.84 (±0.77)x	4.29 (±0.33)x

Values are relative to control cells (=1), normalized to GAPDH. (Means of two to three independent experiments ±SD are shown.)

tors at 96 hours (*Table 3*). Thus, we can conclude that several components of the Notch pathway are transcriptional targets of Notch-signaling itself.

Delta1 treatment had an impact on cell morphology as well (Fig.2.). In mouse cell cultures, control cells exhibited an organized, islet-like pattern on the second day after seeding; this structure was loosened in ligand-treated cells. More strikingly, control rat cells acquired a polygonal shape and were confluent, whereas cells grown on Delta1 retained an elongated, slightly irregular shape, exhibiting a lower cell density and more detached cells than in control cultures.

Discussion

Notch has been widely studied in the context of stem cells, hoping that specific modulation of the signal may provide an *in vitro* tool for stem cell expansion and for the manipulation of lineage specific differentiation in the future. This may be of special interest in MAPCs, where – in particular for populations that express relatively low levels of the ESC transcription factor POU5F1 – large-scale culturing has been difficult because of the requirement of low cell density.

We demonstrated that most Notch-signaling components are present in mouse and rat low-Oct4-MAPCs. Presenilins, Notch1, 2 and 3 receptors, Jagged1 (and to a lesser extent, Jagged2 and Delta1 ligands), CBF1, the target gene HES-1 and regulator molecules such as Numb, Lunatic Fringe and Radical Fringe were all detected; thus, it can be concluded that MAPCs possess the necessary elements required for a functional and fine-tunable Notch signal. In line with our results on rodent cells, human MAPCs have previously also been shown to express substantial Notch1,2,3 and Jagged1, but little Delta1 and Jagged2; however, human MAPCs also expressed some Delta4.[39] In comparison, we found that mouse ESCs expressed substantially higher levels of Notch3 and lower levels of Notch2 than mouse low-Oct4-MAPCs, which corresponds well with previously published results, where Notch1 and 3, and lower levels of Notch2 were reported in ESCs.[40,16] These data suggest that, although stem cells share a number of functioning genes which contribute to their "stemness,[41] these characteristic signatures may vary to some extent according to stem cell type.

Cell density had an impact on the expression of some signaling elements in low-Oct4 rodent MAPCs: Notch3 (as our previous unpublished microarray data suggested) and HERP transcripts increased following culture of low-Oct4 mouse MAPCs for 48-96h at high density, whereas expression of Notch1, Notch2, and – to a lesser extent – Notch3 was increased in low-Oct4 rat MAPCs cultured for 48-96h at high cell density. At least some of these changes are transient, as expression of some of these transcripts returned to baseline levels at 96 hours of culture at high density. The presence or absence of growth factors did not have a consistent impact on the expression of Notch pathway elements, however, they may, in some cases, help sustain cell density dependent transcriptional changes for longer time periods.

Notch signaling may have different target genes in distinct cell types.[4] Immobilized Delta1 initiated signaling through Notch receptors in rodent MAPCs, and in addition to HES-1 (and HERP-1 in mouse cells), expression of Notch1 and Notch3 were increased. Delta1 was also reported to induce HES-1 and Notch3 expression in human CD34+ early hematopoetic cells,[42] which suggests that Notch3 may also be a conserved Notch target in several species and cell types. Whether Notch1 and Notch3 are directly or indirectly regulated by Delta1 has yet to be determined. These transcriptional changes probably comprise a part of a biological feedback loop, which contributes to the tight control of cellular processes.

Notably, HES-1 and Notch1 were present in low density mouse MAPC cultures whereas HERP-1 and Notch3 were expressed at low levels, but all of them were inducible upon stimulation with Delta1. One possibility is that HES-1 expression is maintained by Notch-independent mechanisms when cells have little contact with each other. In fact, the TGFβ, the JNK and the Ras/MAPK signaling pathways were shown to regulate HES-1 in epithelial, endothelial and neuroblastoma cell types, respectively.[43-45] The up-regulation of HERP-1 and other target genes may require Notch-signaling mediated by ligands in the environment, and specific transcriptional changes may depend on signaling strength, time and other factors such as the presence and type of serum.[46]

The role of Notch during differentiation is not yet fully known. Yu et al found that HES-1 expression was up-reg-

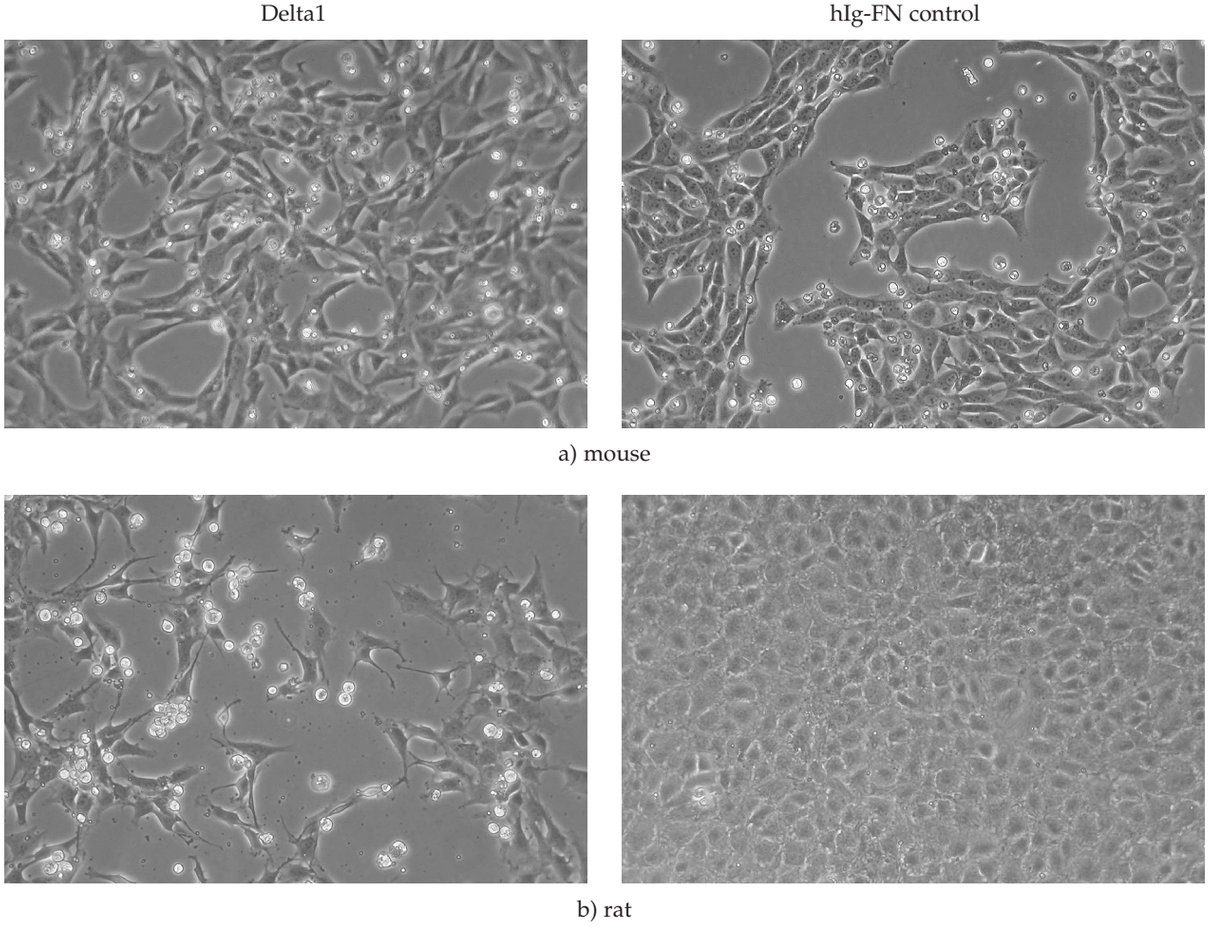

Figure 2. *Morphology of low-Oct4-MAPCs treated with Delta1 ligand for 2 days;* ***(a)*** *mouse,* ***(b)*** *rat cells (magnification: 200x; representative photos are shown).*

ulated in human hematopoetic stem cells compared to more mature progenitors.[47] In contrast, Noggle et al reported the activation of Notch signaling in cells differentiating from human embryonic stem cells.[15] In preliminary experiments, Notch1, Notch3, Notch4, Jagged1, HES-1 and Numb transcripts increased remarkably by day 14 during neuronal differentiation of rat MAPCs, whereas Jagged2 was down-regulated (our unpublished observations), and some of these changes may also be attributable to an increase in Notch activity. Notch activation alone (in the absence of differentiation inducing conditions) was not sufficient to promote differentiation into a specific cell lineage, as determined by screening for the presence of lineage specific transcription factors (our unpublished observations), however, longer time periods may be necessary to detect such changes.

Taken together, we identified the components of the Notch-pathway in rodent low-Oct4 multipotent adult progenitor cells, and showed that the signaling pathway can be modulated in *in vitro* culture systems. Mouse and rat HES-1 and mouse HERP-1 were shown to be transcriptional target genes of the signal in MAPCs. In addition, Notch1 and Notch3 receptors themselves were targets of Notch-signaling in both mouse and rat MAPCs as well. However, the exact transcriptional changes and functional consequences may be species specific. Future studies elucidating the function of Notch in cell fate specification in MAPCs, as well as investigation of Notch signaling pathways in MAPCs expressing different levels of Oct4 will lead to a more thorough understanding of stem cell biology, which – hopefully – will result in the development of practical applications in cell therapy.

Acknowledgement

The Delta-1^{extIgG} ligand was kindly provided by Irwin D. Bernstein (Fred Hutchinson Cancer Center, Seattle, WA, USA). The R1 mouse embryonic cell line was a kind gift from Dr. Janet Rossant (Mount Sinai Hospital, Toronto, Canada). I would like to thank April Breyer and Laura Lien for help with cell cultures and Mark Blackstad for help with PCR.

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