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spring | 2007

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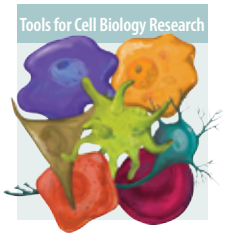
Inside



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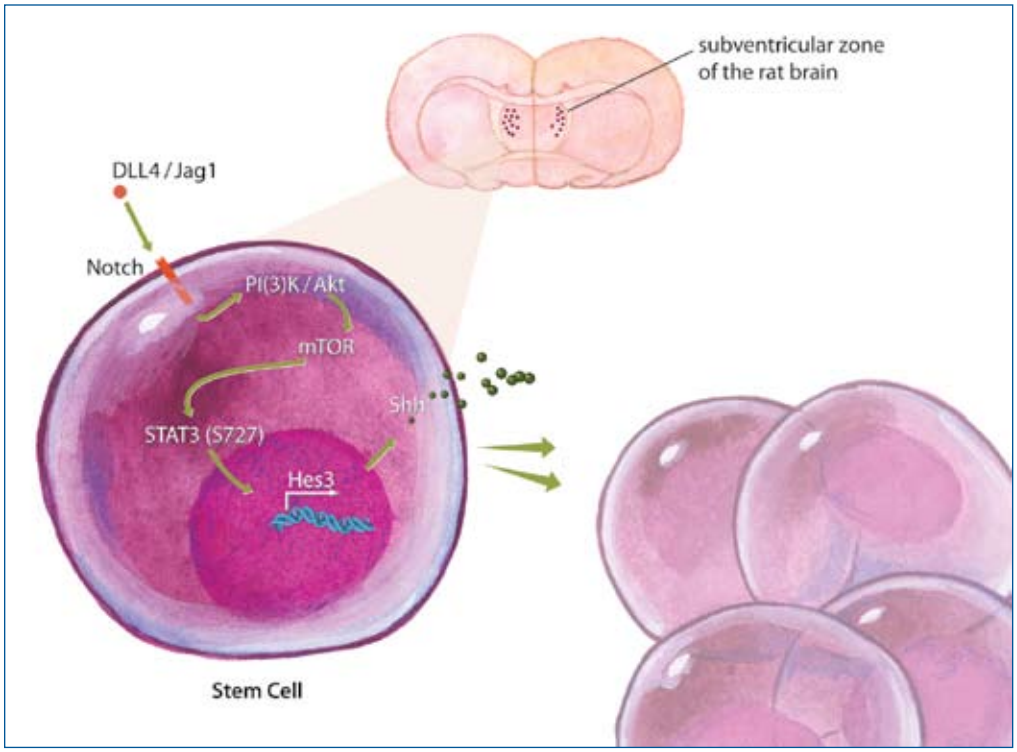

Figure 1. Hypothesized mechanism of Notch-mediated neural stem cell expansion *in vivo*, based on infusion of soluble Notch ligand in the rat model. *In vitro* studies in fetal neural stem cells show that Notch activation by DLL4 induces a cytoplasmic signaling cascade that preserves fetal neural stem cell pluripotency and encourages cell expansion through STAT3-dependent Sonic hedgehog (Shh) release. (adapted from reference 4)

Taking Advantage of Notch in Stem Cell Expansion

The future utilization of stem cell therapy for neurodegenerative disease has been impeded by the limited numbers of stem cells that can be generated *in vitro* for transplantation, the potential immune response to the transplanted cells, and the sparse population of endogenous progenitor cells that exist *in vivo*. Thus, in addition to mastering the isolation and molecular guidance of stem cells, research is focusing on understanding mechanisms that increase stem cell numbers and survival, and minimize host rejection.

Notch encodes a transmembrane receptor with signaling integral to development and cancer. Notch signaling occurs through ligand-induced cleavage that frees the Notch intracellular domain (Nicd) to enter the nucleus and alter gene expression via tissue-specific transcription factors.[1] Among numerous roles in nervous system development, Notch is imperative in stem cell biology because its signaling maintains an undifferentiated progenitor population.[2,3] However, the exact mechanism by which Notch prevents differentiation to prolong self-renewal and pluripotency, and its manipulation for therapeutic applications, has only recently been proposed (Figure 1).

Androutsellis-Theotokis and colleagues outlined a signaling cascade initiated by Notch ligands, Delta-like 4 (DLL4) and Jagged 1 (Jag1), that increased fetal neural stem cell (NSC) survival without inducing differentiation *in vitro*.[4] This pathway includes the obligate cleavage of Nicd. However, the rapid timeline of cell expansion indicates that Nicd influences cytoplasmic effectors rather than, or in addition to, the commonly-associated yet delayed transcriptional response. Examination of kinase activation and administration of JAK and p38 kinase inhibitors identified that DLL4 and Jag1 led to activation of Akt kinase and mammalian target of rapamycin (mTOR). Both are serine/threonine kinases related to cell proliferation and differentiation, with downstream phosphorylation of transcription factor STAT3.

Other research has associated Notch signaling and STAT3 activation with differentiation to a glial cell fate.[5-7] This is in apparent contrast to a Notch-dependent preservation of pluripotency. The discrepancy may be explained by different phosphorlyation sites. Rather than the previously reported phosphorlyation of STAT3 (Tyr705) that drives glial cell fate,[7] Androutsellis-Theotokis *et al.*

Notch continued on page 5

MMP-12 in Smoking-Related Pulmonary Disease

Chronic obstructive pulmonary disease (COPD), the fifth leading cause of death worldwide, is estimated by the World Health Organization (WHO) to affect 80 million people.[1] Cigarette smoking is the major risk factor for COPD, which includes both emphysema and chronic bronchitis, also called small airway disease. In emphysema, peripheral air spaces in the lung are enlarged and walls of bronchioles and alveoli are destroyed. Chronic bronchitis, which may occur concurrently with emphysema, includes airway wall repair-induced fibrosis.[1] Inflammation, proteinase imbalance, oxidative stress, and apoptosis all appear to be interwoven in the pathogenesis of COPD, and the matrix metalloproteinase MMP-12 plays a role in each of these processes (Figure 1).

Disruption of the balance between proteolytic enzymes, such as elastases and their inhibitors, has long been considered the major cause of COPD.[2] This protease-antiprotease imbalance is now thought to be caused by chronic inflammation.[2] Macrophage elastase MMP-12 (inhibited by TIMP-1) and neutrophil elastase (inhibited by α-1-antitrypsin) are the most abundant elastases in the lung. TNFα, which is released from the cell membrane by MMP-12, is the major recruiter of neutrophils to the lung; IFN-γ also recruits neutrophils and stimulates MMP-12 activity.[2-4] Once present, neutrophil elastases and oxidants secreted in the inflammatory environment mediate much of the destruction of lung tissue.[5] Elastin fragments are also chemotactic, recruiting monocytes that differentiate to form alveolar macrophages that comprise the bulk of the inflammatory cells accumulating in the interstitium, septum, and alveolar airspaces in emphysema.[6]

Some elements of COPD pathogenesis precede macrophage or neutrophil accumulation. Studies on airway cells *in vitro* bypass the influence of inflammatory cells and show direct effects of smoke or smoke condensates. For example, the oxidative effects of smoke induce MMP-12 expression via a TNF-α-dependent pathway in cultured airway-like epithelia.[7] Oxidants are also involved in release of TGF-β from latency in tracheal explants.[8] Active TGF-β is likely to mediate fibrogenic airway remodeling, an effect that is blocked in MMP-12-deficient mice.[8] Similarly, apoptotic pathways induced by either TGF-β or Fas (CD95) may cause lung fibrosis that can be ameliorated by deletion of MMP-12.[9,10] Human airway smooth muscle cells have also been shown to contribute active MMP-12 in response to IL-1β and TNF-α.[11]

A pivotal study showed that deletion of mouse MMP-12 abrogates development of cigarette smoke-induced COPD.[12] Since then, other studies have shown that deletion or inhibition of neutrophil elastase, TGF-β, or TNF-α substantially reduces lung damage in response to cigarette smoke.[3,4,7,8,13] Conversely, naturally occurring human deficiency of α-1-antitrypsin, the major human inhibitor of neutrophil elastase, confers susceptibility to COPD.[1,2] It is clear that the entire story of COPD pathogenesis involves these and other players in a complex, interwoven cascade. The development of COPD in response to cigarette smoke is not universal and, when it does occur, varies in time of onset for both humans and rodent strains.[1,2] This variation likely results from interaction of genetic influences with environment. One example is the increased susceptibility to COPD in smokers that have specific polymorphisms in both human MMP-1 and MMP-12.[14]

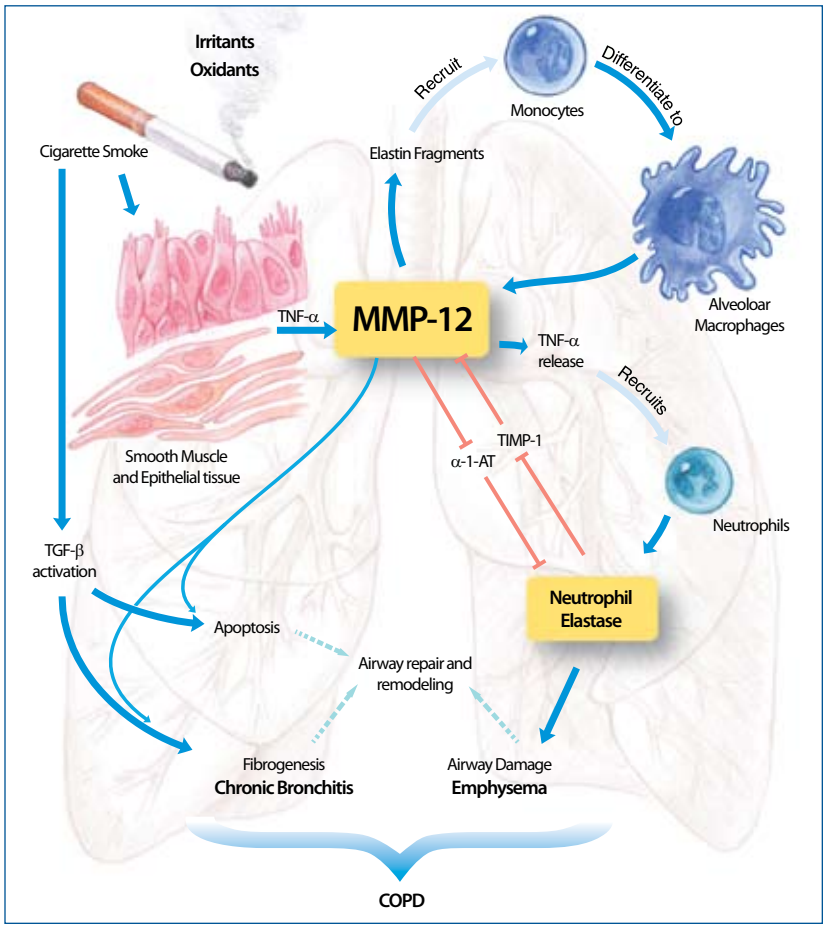

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Figure 1. MMP-12 is produced by alveolar macrophages, smooth muscle cells, and epithelia in response to cigarette smoke. It is a key molecule in the recruitment of inflammatory cells, release of TNF-α, and pathways downstream of TGF-β activation. These activities lead to the airway damage, fibrogenesis, repair, and remodeling that are the hallmarks of COPD.

Regulation of VE-Cadherin and VEGF R2 by VEGF

VE-Cadherin and VEGF R2 are transmembrane glycoproteins that are expressed in the adherens junctions between vascular endothelial cells (EC). VE-Cadherin interacts homophilically between neighboring cells to provide strength to the endothelium. VE-Cadherin adhesion is reduced during angiogenesis and leukocyte extravasation, two processes that require decreased EC attachment. It is well established that adherens junction integrity is regulated by VE-Cadherin phosphorylation and internalization in response to VEGF stimulation.[1,2] Several recent publications provide additional details about the molecular mechanism directing this process.

In adherens junctions between quiescent vascular EC, VEGF R2 is maintained in an inactive state by protein tyrosine phosphatases.[2] VEGF binding activates the tyrosine kinase domain of VEGF R2, initiating the sequential activation of the Src-Vav2-Rac1-PAK pathway, which results in the phosphorylation of VE-Cadherin at Ser665 by PAK.[3] The subsequent binding of β-arrestin2 to serine-phosphorylated VE-Cadherin promotes the internalization of VE-Cadherin into clathrin-coated pits.[3] This disrupts the architecture of endothelial junctions and allows for the passage of molecules and cells. In addition, phosphorylation of the VE-Cadherin complexes by Src at Tyr685 may contribute to the disassembly of adherens junctions.[4]

It was recently reported that VEGF R2 signaling is enhanced by endocytosis. Interestingly, VE-Cadherin was shown to play an inhibitory role in this process. By binding to VEGF R2, VE-Cadherin prevents VEGF R2 endocytosis. This favors inactivation by a junction-associated, transmembrane tyrosine phosphatase, called DEP-1.[5] When clathrin-dependent internalization transports phosphorylated VEGF R2 to endosomal vesicles, uninterrupted VEGF R2 signaling is made possible by phosphorylated Tyr1175 inaccessible to cell surface DEP-1.[5] Phosphorylated Tyr1175 is required for the binding and activation of PLCγ, a primary mediator of VEGF R2-promoted cell proliferation.[6] Notably, cell surface VEGF R2-mediated clathrin-dependent internalization delivers VE-Cadherin to compartments distinct from intracellular VEGF R2 compartments.[3]

Other molecules influence these processes as well, although specifically how they are integrated into the above pathways is not clear. The scaffolding protein IQGAP1, which is bound to VE-Cadherin both at the adherens junction and after internalization, is necessary for VE-Cadherin localization at cell-cell contacts.[7] It potentially mediates the interaction of VE-Cadherin and VEGF R2, and also tethers VE-Cadherin to the cytoskeleton.[1,7] In addition, VEGF stimulation of quiescent microvascular EC induces the transport of intracellular JAM-C to adherens junctions, where it disrupts VE-Cadherin adhesion.[8] TNF-α also promotes vascular permeability by inducing Fyn-dependent tyrosine phosphorylation of VE-Cadherin.[9] Lastly, exposure of endothelium to oxidized LDL promotes VE-Cadherin internalization and increased monocyte transendothelial migration.[10]

These advances in the understanding of the interactions between VE-Cadherin and VEGF R2 clarify how the adherens junction is regulated in response to VEGF stimulation. Prolonged VEGF R2 signaling enables the continued proliferation and migration of vascular EC during angiogenesis. Internalization of VE-Cadherin weakens adherens junction adhesion, permitting increased EC mobility, vascular permeability, and leukocyte extravasation.

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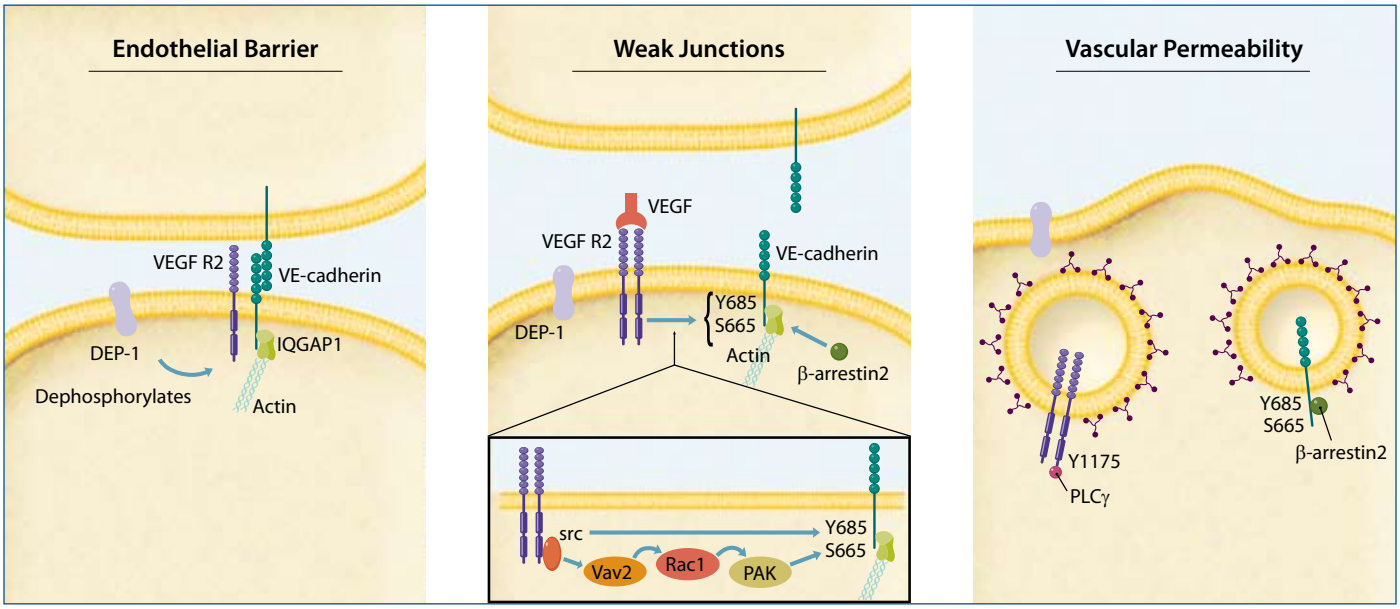

Figure 1. Homophilic interactions between VE-cadherin molecules at adherens junctions in adjacent endothelial cells help maintain the endothelial barrier. Upon VEGF exposure, activation of VEGF R2 triggers a phosphorylation cascade that targets VE-cadherin. VEGF R2 continues to signal after internalization, while sequestration of VE-cadherin in endosomes disrupts adhesion. This process promotes loss of cell-cell contacts, increased vascular permeability, and endothelial cell migration. (Figure adapted from those contained within references 2 & 3)

Sonic Hedgehog: a morphogen involved in axon guidance

A morphogen is classically defined as a signaling molecule that elicits different cellular responses depending on its concentration. More specifically, morphogens are secreted molecules that drive the organization of regional groups of cells into patterns. The absolute concentration of the morphogen acting on any one cell determines developmental fate.[1,2] An axon guidance molecule, by contrast, has only one function – to either attract or repel a motile growth cone. In contrast to morphogens, their guidance function is determined by a cell's ability to detect a change in guidance molecule concentration over distance. Until recently, morphogens and guidance molecules were considered structurally and functionally distinct. Now, however, it would appear that select, early-expressed morphogens can be temporally "recycled" and serve as axon guidance cues. Sonic hedgehog (Shh), along with members of the Wnt and bone morphogenetic protein (BMP) families, is a molecule that acts early as a morphogen to determine neuronal fate and later as an axon guidance factor to help direct the paths of developing neurons (Figure 1).[3-6]

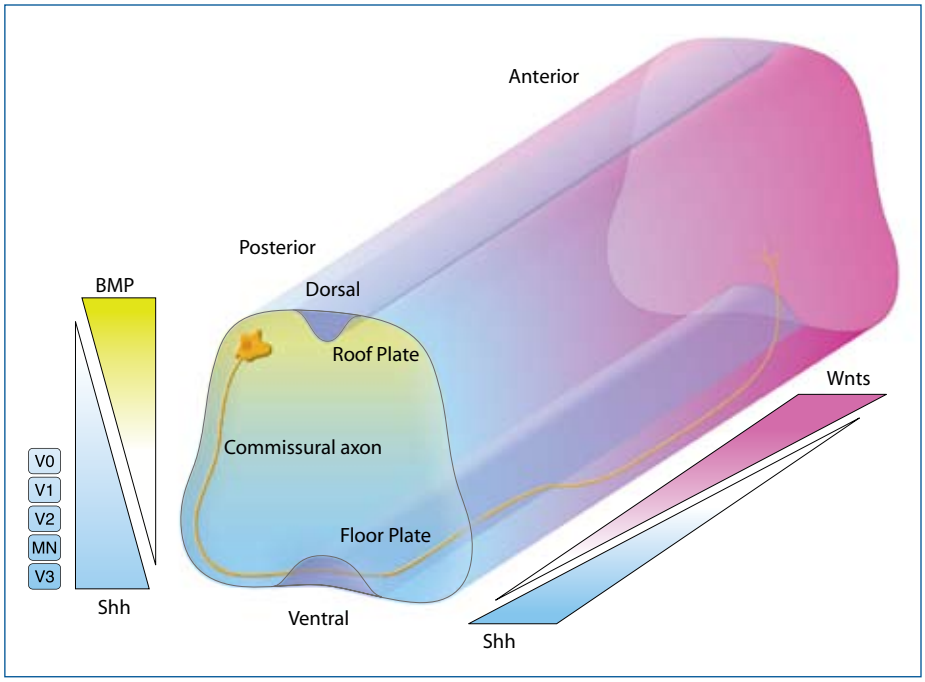

Figure 1. A gradient of Shh acts in the early neural tube to specify ventral neurons (V0-V3 and MN), while a BMP gradient specifies dorsal neurons. Vertebrate commissural neurons of the dorsolateral spinal cord take a ventral trajectory toward the floor plate, cross the midline, and then turn, following along the floor plate, while moving anteriorly towards the brain. Shh, BMP, and Wnt are morphogens that help guide commissural axons along this path.

As a morphogen, Shh participates in the patterning of the developing spinal cord. Following closure of the neural tube, commissural (crossing; contralateral) and association (same side; ipsilateral) neurons develop in the dorsal half of the cord, while inter- and motor-neurons form in the ventral half of the cord. Shh is secreted by the ventral floor plate. As the concentration of Shh diminishes dorsally, at least five distinct neuron cell types form along its gradient, demonstrating morphogenic ability. Four interneuron cell types (termed V0-V3) plus lower motor neurons (MN) are induced through Shh-mediated activation and repression of homeodomain transcription factors. Based on the level of Shh concentration, different thresholds for repression and activation of the transcription factors give rise to a "code" of progenitor domains.[7] Thus, neurons generated in the more ventral progenitor domains, nearest the floor plate, result from higher levels of Shh.[7,8]

As an axon guidance molecule, Shh impacts the positioning of dorsal commissural axons following its morphogenic effects on neuron formation. Initially, BMP-7, produced dorsally, prohibits developing dorsal axons from crossing the midline in the region of the roof plate.[7,9] This leaves only a ventral direction for growth. Once they have arrived at their ventral location, both Shh and Netrin-1 collaborate to chemoattract dorsal axons towards the midline.[10] Remarkably, following crossing of the midline, Shh acts as a chemorepellent rather than chemoattractant. Shh, together with an anterior-to-posterior Wnt gradient plus an Ephrin B signaling cascade, directs neurons to turn at a 90° angle and continue migration in the anterior direction towards the brain.[11-14]

Distinct receptors on axons help mediate the differential responses to Shh. It is suggested that BOC (Brother of CDO), working in concert with Smoothened, mediates chemoattraction, while a temporally-regulated Hip (Hedgehog Interacting Protein) receptor mediates chemorepulsion.[4,13,15] Moreover, morphogenic functions of Shh to determine neuronal cell fate are thought to signal through the Patched (ligand binding) and Smoothened (signal transducing) receptors.[7]

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RECENT CITATIONS: Leptin & Leptin Receptor

1. Blardi, P. *et al.* (2007) *Rett syndrome and plasma leptin levels.* J. Pediatr. **150**:37.

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   Application: ELISA

2. Chen, K. *et al.* (2006) *Induction of leptin resistance through direct interaction of C-reactive protein with leptin.* Nat. Med. **12**:425.

   Mouse Leptin Quantikine ELISA Kit (Catalog # MOB00)

   Sample Type: Mouse Serum

   Application: ELISA

3. Crandall, D.L. *et al.* (2006) *Modulation of adipose tissue development by pharmacological inhibition of PAI-1.* Arterioscler. Thromb. Vasc. Biol. **26**:2209.

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   Sample Type: Human Preadipocyte Supernatant

   Application: ELISA

4. De Rosa, V. *et al.* (2007) *A key role of leptin in the control of regulatory T cell proliferation.* Immunity **26**:241.

   Human Leptin Quantikine ELISA Kit (Catalog # DLP00)

   Sample Type: Human T_{reg} and $CD4^+CD25^-$ T Cells

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   Application: Bioassay – Cell Stimulation

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   Mouse Recombinant Leptin Receptor (Catalog # 497-LR)

   Sample Type: $CD4^+$ T Cells

   Application: Bioassay – Neutralization of Leptin; Production and Purification of Mouse polyclonal Ab

6. Fenton, J. *et al.* (2007) *Leptin induces an Apc genotype-associated colon epithelial cell chemokine production pattern associated with macrophage chemotaxis and activation.* Carcinogenesis **28**:455.

   Mouse Recombinant Leptin (Catalog # 498-OB)

   Sample Type: Preneoplastic & Normal Colon Epithelial Cell Lines

   Application: Bioassays—Cell Proliferation, Cytokine Antibody Array, Macrophage Chemotaxis, & Macrophage Activation/Nitric Oxide Assay

   Mouse Leptin Quantikine ELISA Kit (Catalog # MOB00)

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   Application: ELISA

7. Kos, K. *et al.* (2007) *Adiponectin and resistin in human cerebrospinal fluid and expression of adiponectin receptors in the human hypothalamus.* J. Clin. Endocrinol. Metab. **92**:1129.

   Human Leptin Quantikine ELISA Kit (Catalog # DLP00)

   Sample Type: Human Serum and CSF

   Application: ELISA

   Human Resistin Quantikine ELISA Kit (Catalog # DRSN00)

   Sample Type: Human Serum and CSF

   Application: ELISA

8. Macia, L. *et al.* (2006) *Impairment of dendritic cell functionality and steady-state number in obese mice.* J. Immunol. **177**:5997.

   Mouse Leptin DuoSet® ELISA Kit (Catalog # DY498)

   Sample Type: Mouse Serum

   Application: ELISA

9. Stefanou, N. *et al.* (2006) *Leptin receptor isoforms mRNA expression in peripheral blood mononuclear cells from patients with chronic viral hepatitis.* Exp. Biol. Med. **231**:1653.

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   Sample Type: PBMCs

   Application: Flow Cytometry

10. Steinberg, G.R. *et al.* (2006) *Ciliary neurotrophic factor suppresses hypothalamic AMP-kinase signaling in leptin-resistant obese mice.* Endocrinology **147**:3906.

    Mouse Recombinant Leptin (Catalog # 498-OB)

    Sample Type: *In Vivo* Injection

    Application: Bioassay – Effect on Food Intake and Body Mass

11. Tukiainen, E. *et al.* (2006) *Leptin and adiponectin levels in acute pancreatitis.* Pancreas **32**:211.

    Human Leptin Quantikine ELISA Kit (Catalog # DLP00)

    Sample Type: Human Plasma (Citrate)

    Application: ELISA

    Human Adiponectin Quantikine ELISA Kit (Catalog # DRP300)

    Sample Type: Human Plasma (Citrate)

    Application: ELISA

Notch continued from page 1

discovered that phosphorylation of STAT3 on Ser727 is integral to Notch-dependent cell expansion through its upregulation of both the Hes3 transcriptional repressor and expression of Sonic hedgehog (Shh).[4] To further support the critical contribution of Notch to NSC, Basak and Taylor generated transgenic mice expressing Hes5-GFP under a Notch1 reporter and correlated the degree of self-renewal and pluripotency *in vitro* to the level of Notch1 activity in the developing nervous system.[8] In combination, these results unveil the multiple paths of Notch influence on stem cell integrity and fate, thus providing molecular targets to manipulate stem cell populations.

To support their *in vitro* findings, Androutsellis-Theotokis *et al*. infused DLL4 and/or FGF basic into the normal adult rat brain. While FGF basic had little effect, DLL4 stimulated the proliferation of a subset of cells that expressed early neuronal markers, but lacked markers of mature glial cells and neurons.[4] Supplementing previous studies that examined Notch signaling in experimental brain injury,[9,10] rats subjected to ischemic injury followed by DLL4 and FGF basic treatment exhibited improved motor recovery at 45 days post-injury.[4] These results suggest that exogenous stimulation of Notch signaling enhances the endogenous progenitor cell population. This highlights a potential therapeutic approach to maximize an innate capacity for self-repair.

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ENDO – The Endocrinology Society Booth #1611
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International Society for Stem Cell Research (ISSCR) Booth #39
Queensland, Australia .....June 17-20, 2007

NEW TOOLS: Recombinant Proteases of the Renin-Angiotensin System (RAS)

The Renin-Angiotensin System (RAS) plays a critical role in maintaining blood pressure homeostasis as well as fluid and salt balance in mammals. Production of angiotensins from angiotensinogen requires the participation and coordination of many proteases in different pathways (Figure 1).[1-7] R&D Systems now offers RAS-related recombinant proteases that can be used in studies of their structure-function relationship and in development of their activators and inhibitors.

R&D Systems offers recombinant proteases of RAS

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Renin	Human Renin	4090-AS	Pro	Trypsin 3 (3714-SE)	RE(EDANS)IHPFHLVIHTK (DABCYL)R
Angiotensin I-converting enzyme	Human ACE Mouse ACE	929-ZN 1513-ZN	Ecto Ecto		Mca-RPPGFSAFK(Dnp)-OH (Catalog # ES005)
Angiotensin I-converting enzyme 2	Human ACE2 Mouse ACE2	933-ZN 3437-ZN	Ecto Ecto		Mca-YVADAPK(Dnp)-OH (Catalog # ES007)
Chymase	Human CMA1	4099-SE	Pro	Cathepsin C (2336-CY)	Suc-AAPF-AMC
Cathespin A	Human CTSA Mouse CTSA	1049-SE 1123-SE	Pro Pro	Cathepsin L (952-CY)	Mca-RPPGFSAFK(Dnp)-OH (Catalog # ES005)
Aminopeptidase A	Human APA Mouse APA	2499-ZN 2809-ZN	Mature Mature		E-AMC
Aminopeptidase N	Human APN Mouse APN	3815-ZN 2335-ZN	Ecto Ecto		A-AMC
Neprilysin	Human NEP Mouse NEP	1182-ZN 1126-ZN	Ecto Ecto		Mca-RPPGFSAFK(Dnp)-OH (Catalog # ES005)
Thimet oligopeptidase	Human THOP1	3439-ZN	Full length		Mca-PLGPK(Dnp)-OH
Neurolysin	Human NLN	3814-ZN	Full length		Mca-PLGPK(Dnp)-OH

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Proteases of RAS

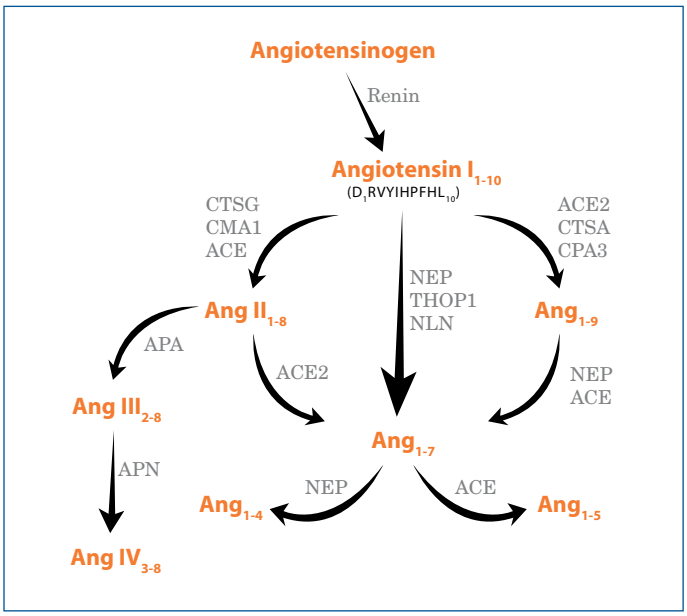


Figure 1. A cascade of proteolytic reactions in RAS results in the generation of different angiotensin (Ang) peptides. Renin cleaves the precursor protein, angiotensinogen, releasing the inactive peptide, angiotensin I. The Carboxypeptidases ACE, ACE2, CMA1, CTSA and CPA3, the aminopeptidases APA and APN, and the endopeptidases CTSG, NEP, THOP1 and NLN participate in various pathways to generate Ang peptides with diverse functions. For example, Ang II and III act as vasoconstrictors whereas Ang_{1-7} acts as a vasodilator. Active Ang peptides function through their respective receptors, and the same Ang peptides may have different effects through different receptors (not shown).

Optimization of Chymase Activation

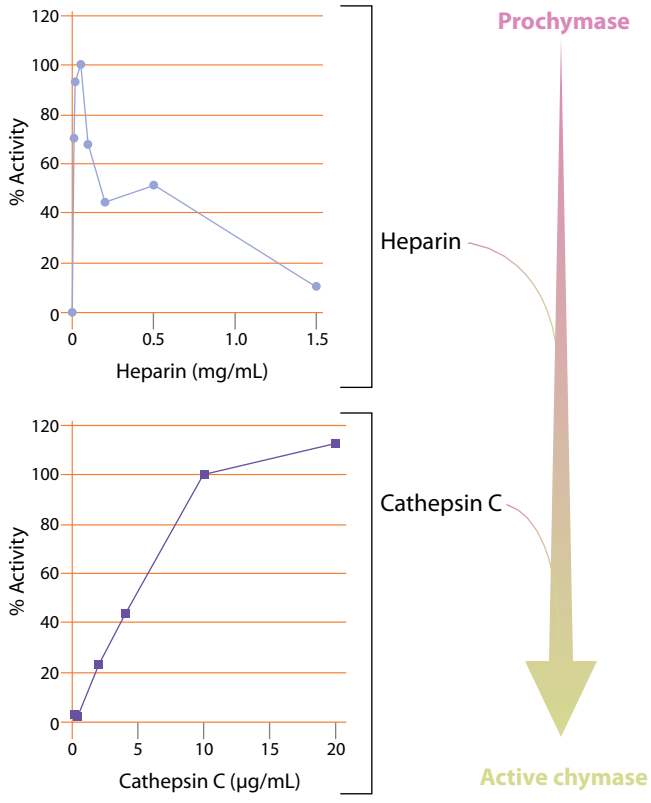


Figure 2. The conversion of prochymase into active chymase is mediated by recombinant mouse Cathepsin C (Catalog # 2336-CY) in the presence of heparin. The optimal concentrations of heparin and Cathepsin C were obtained by fixing Cathepsin C (10 µg/mL, top graph) or heparin (0.05 mg/mL, bottom graph).

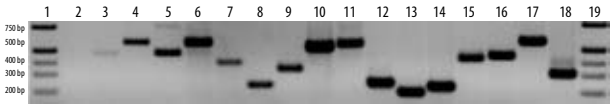
TECHNICAL NOTES: Assessing the Pluripotent Status of Stem Cells

Embryonic stem cells have the potential to differentiate into multiple cell types and are widely recognized as holding significant promise for therapeutic applications. The quality of a stem cell culture determines its ability to give rise to differentiated cell types efficiently, and can be measured by analysis of the expression of molecular markers of the pluripotent phenotype. Markers of early lineage committed cells are also useful to determine whether differentiated cells are present in a stem cell culture, and can indicate which lineage pathway the differentiated cells represent.

The R&D Systems Human Pluripotent Stem Cell Assessment Primer Pair Panel (Catalog # SC012; Figure 1) profiles the mRNA transcripts of fourteen genes that are frequently used as markers for molecular characterization of undifferentiated and early lineage-committed human ES cells. A primer pair for human GAPDH is included and can be used as a control for successful cDNA synthesis. A positive control is also included. In addition, R&D Systems offers a mouse/rat version of this kit (Catalog # SC015).

Additional reagents to assess stem cell phenotype are available from R&D Systems. For example, Oct 3/4 expression is detected in undifferentiated NTera2 human teratocarcinoma cells by flow cytometry (Figure 2), and DPPA4 expression is shown by immunocytochemistry in the nuclei of mouse D3 embryonic stem cells (Figure 3).

Human Pluripotent Stem Cell Assessment Primer Pair Panel



Analytes

1. DNA marker
2. Negative control
3. DPPA5/ESG1
4. Nanog
5. Oct3/4
6. SOX2
7. Nestin
8. Otx2
9. TP63
10. AFP
11. GATA-4
12. PDX-1
13. SOX17
14. HNF-3β
15. Brachyury
16. Stella
17. GAPDH
18. Positive control
19. DNA marker

Figure 1. PCR products amplified by primer sets specific for 14 different markers of stem cell pluripotency and lineage commitment visualized by gel electrophoresis. cDNAs used for amplification were derived from a variety of embryonic tissues. Markers in lanes 3-6 are expressed in undifferentiated ES cells, lanes 6-9 ectodermal lineage, lanes 10-14 endodermal lineage, lane 15 mesodermal lineage, and lane 16 contains a marker for germ cells. Lanes 1, 2, and 17-19 show controls.

Oct3/4 Detection by Flow Cytometry

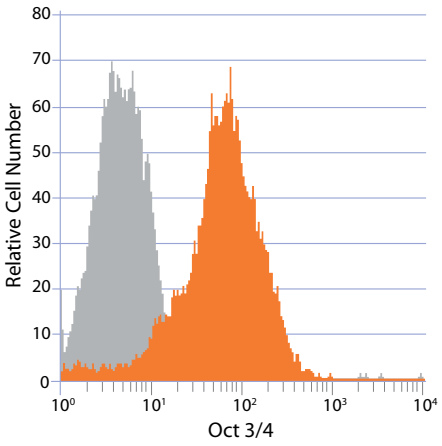


Figure 2. Assessment of Oct3/4 expression in NTera2 cells by intracellular flow cytometry using rat anti-human/mouse Oct3/4 monoclonal antibody (Catalog # MAB1759; orange) or isotype control (Catalog # MAB0061; gray) followed by APC-conjugated secondary antibody.

DPPA4 in Pluripotent Mouse ES Cells

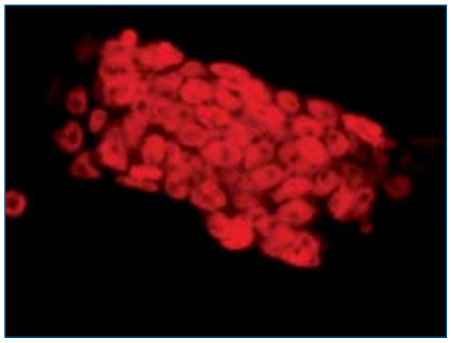


Figure 3. Detection of DPPA4 in the nuclei of D3 cells using R&D Systems anti-mouse DPPA4 polyclonal antibody (Catalog # AF3730) and R&D Systems Northern-Lights™ 557-conjugated secondary antibody (Catalog # NL001).

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