

Purines, Purinergic Receptors, and Cancer

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Abstract

Purines were long thought to be restricted to the intracellular compartment, where they are used for energy transactions, nucleic acid synthesis, and a multiplicity of biochemical reactions. However, it is now clear that both adenosine and adenosine triphosphate are (i) abundant biochemical components of the tumor microenvironment, (ii) potent modulators of immune cell responses and cytokine release, and (iii) key players in host–tumor interaction. Moreover, both ATP and adenosine directly affect tumor cell growth. Adenosine is a powerful immunosuppressant (mainly acting at A2A receptors) and a modulator of cell growth (mainly acting at A3 receptors). ATP is a proinflammatory (acting at P2Y1, P2Y2, P2Y4, P2Y6, and P2Y12, and at P2X4 and P2X7 receptors), an immunosuppressant (acting at P2Y11), and a growth-promoting agent (acting at P2Y1, P2Y2, and P2X7 receptors). This complex signaling network generates an array of inhibitory and stimulatory responses that affect immune cell function, tumor growth, and metastatic dissemination. Investigation of purinergic signaling has increased our understanding of the tumor microenvironment and opened new and exciting avenues for the development of novel therapeutics. *Cancer Res*; 72(21); 5441–7. ©2012 AACR.

Extracellular Purines: A Heterodox Concept

ATP, the key intracellular energy currency, was isolated by Karl Lohmann in 1929 (1). At about the same time, Drury and Szent-György reported the first effects of extracellular purines on cell responses (2). However, and with hindsight rather surprisingly, the observation of Drury and Szent-György was neglected for several decades, until the role of extracellular purines as extracellular messengers was brought to light by the seminal contributions of Robert Berne, Bertil Fredholm, and above all Geoff Burnstock (3–5). Indeed, we owe our present understanding of purinergic signaling and the current classification of P2 receptors to Geoff Burnstock's stubborn activity and enthusiasm (6). Although other nucleotides, such as the pyrimidines UTP and UDP, have important extracellular signaling roles, most recent reports have highlighted the involvement of ATP in host–tumor interaction. As for nucleosides, there is no convincing demonstration that other nucleosides besides adenosine participate in cell-to-cell signaling; thus in this review, I concentrate on ATP and adenosine.

Adenosine receptors, named P1, comprise 4 members, A1, A2A, A2B, and A3, which couple via different G proteins to phospholipase C and adenylyl cyclase. Other intracellular transduction systems, such as the mitogen-activated protein kinase pathway, can also be triggered (7). Nucleotide receptors, named P2, comprise 2 subfamilies, metabotropic P2Y and

ionotropic P2X receptors (8). P2Y receptors can be further subdivided into 2 groups depending on the coupling to specific G proteins: P2Y1, P2Y2, P2Y4, and P2Y6 couple to Gq to activate phospholipase C β , whereas P2Y12, P2Y13, and P2Y14 couple to Gi to inhibit adenylyl cyclase. P2Y11 is peculiar in that it couples to both Gq and Gs and thus triggers an increase in intracellular Ca²⁺ and in cAMP levels. P2X receptors are cation-selective, ATP-gated plasma membrane channels made by the assembly of 7 different subunits (P2X1–7) to form trimeric receptors (9). P2X1, 2, 3, 4, 6 subunits can assemble to form homotrimeric or heterotrimeric channels, whereas P2X5 is poorly active as a homomeric complex and seems to be functional mainly as a P2X1/P2X5, P2X2/P2X5, or P2X4/P2X5 heterotrimer (10). Intriguingly, the P2X2/P2X5 heterotrimer exhibits functional features, notably large pore formation, to date thought to be hallmarks of P2X7. The P2X7 subunit, on the other hand, assembles as a homotrimer and, under certain conditions, as a homoexamer (11, 12). For all P2X receptors, the main signal-transducing mechanism seems to be the alteration in the intracellular ion concentration, but in addition, P2X7 has been reported to interact directly with at least 11 different intracellular proteins, among which are heat shock proteins, β -actin, and phosphatidylinositol 4-kinase (13).

P2Y and P2X receptors are widely distributed across animal species and ubiquitously present on both excitable and non-excitable mammalian cells. Within the P2X subfamily, the P2X4 and P2X7 subtypes are expressed to high level by immune cells (14). Participation of P2X4 to immune responses is as yet not well characterized, whereas on the contrary, P2X7 has a role in interleukin-1 β (IL-1 β) release, Ag presentation, and lymphocyte proliferation and differentiation (15, 16). P2Y and P2X receptors significantly differ in their ligand selectivity, because whereas P2Y receptors recognize a wide range of purinergic agonists, ATP is the only physiologic ligand of P2X receptors so far identified. At P2Y1, P2Y12, and P2Y13 receptors, the

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doi: 10.1158/0008-5472.CAN-12-1600

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preferred ligand is ADP; at P2Y2, UTP and ATP are equally active; at P2Y4, the preferred agonist is UTP; at P2Y6, UDP; and at P2Y11, ATP. Physiologic ligands at P2Y14 are sugar nucleotides such as UDP-glucose and UDP-galactose. The presence of 15 (8 P2Y and 7 P2X) different P2 receptor subtypes with different nucleotide selectivity, widely different affinity, and the ability to form different heteromeric channels confers to purinergic signaling a unique plasticity that allows modulation of cell functions by even minute changes in the local extracellular nucleotide concentration. This unusual plasticity is reflected in the ability to finely tune physiologic and pathophysiological responses as different as neurosecretion, coagulation, smooth-muscle contraction, cell growth and apoptosis, or inflammation. Host-tumor interactions should now be included in this list.

Ligand affinity of P2 receptors ranges from the nanomolar (e.g., P2Y2) to the hundred micromolar (e.g., P2X7) level. P1 receptors' affinity for adenosine ranges from the low (A1, A2A, A3) to the high nanomolar (A2B) level. P2 and P1 receptors, in response to changes in the local nucleotide and/or nucleoside concentration, mediate chemotaxis and retention of immune cells at inflammatory sites, phagocytosis and cytokine release, proliferation, or cytotoxicity (4, 17). Moreover, nucleotides also play a crucial role in blood coagulation, because ADP acting at P2Y12 is a potent trigger of platelet aggregation, and ATP acting at P2X7 is a trigger of tissue factor release (18, 19). Purinergic signaling is also modulated by cross-talk between P1 and P2 receptors or by the formation of A2A/P2Y heterodimeric receptors (20).

The Tumor Microenvironment Is ATP and Adenosine Rich

ATP and adenosine accumulate within the tumor microenvironment, which should not be surprising given the established role of ATP as a danger signal and a proinflammatory mediator. It is well known that malignant tumors trigger a strong inflammatory response and are frequently characterized by formation of diffuse necrotic foci. Under these conditions, ATP accumulates into the extracellular space as a result of either leakage due to nonspecific membrane damage or efflux through specific permeation pathways. However, recent findings show that ATP release is neither the mere epiphenomenon of damage to tumor or host stromal cells nor a byproduct of inflammatory cell activation, but rather a process closely involved in cancer cell metabolism and in antitumor immunity (21). Whether extracellular ATP accumulation will turn out to be beneficial or detrimental for the host will depend on the concentration, the rate of degradation to adenosine, and the panel of P2 receptors expressed by the tumor cells and by the infiltrating inflammatory cells.

ATP concentration in the extracellular space is the net result of release and degradation. In addition, synthesis of ATP by ecto-adenylate kinase or nucleotide diphosphate kinase has also been described (22). This latter process is likely to have an important role in keeping extracellular ATP concentration constant, but its precise role in purinergic signaling is incompletely understood. ATP degradation occurs via the sequential

activity of 4 different ectonucleotidase subtypes: ectonucleotide pyrophosphatase, ectonucleotide triphosphate diphosphohydrolase (E-NTPDase), alkaline phosphatase, and ecto-5'-nucleotidase (23). The end product is adenosine, which is finally degraded to inosine by adenosine deaminase. Two members of the ectonucleotidase family seem to have a crucial role in tumor-immune cell interaction, the E-NTPDase CD39 and the 5'-nucleotidase CD73, which are both expressed on the surface of infiltrating immune cells and are the main pathways for the generation of extracellular adenosine.

Presence of specific receptors and efficient degrading systems on the cell surface is a strong hint of, but by no means unequivocal evidence for, the presence of ATP in the extracellular space and for a signaling role of this nucleotide. An important step forward in understanding the signaling role of ATP has been made with the recent development of plasmamembrane luciferase (pmeLUC), a probe for the measurement of the extracellular ATP concentration *in vivo* (24). PmeLUC is a modified luciferase, targeted and localized to the plasma membrane, with the ATP-binding site facing the extracellular space. This plasmamembrane-expressed luciferase allows real-time *in vivo* measurement of ATP in the pericellular space with high sensitivity, specificity, and reproducibility. PmeLUC can be transfected into reporter cells that are then inoculated into tumor-bearing mice to probe the ATP concentration of the tumor microenvironment (25). Alternatively, pmeLUC can be transfected into the tumor cells themselves to directly monitor changes in the ATP concentration of the microenvironment during tumor progression (26). Thanks to pmeLUC, we were able to show that the extracellular ATP concentration is in the hundred micromolar range in the tumor extracellular milieu, whereas it is undetectable (submicromolar) in healthy tissues (25). ATP levels within the tumor microenvironment are sufficient to stimulate even the low affinity P2X7 receptor.

ATP can be released from the infiltrating inflammatory cells as well as from the tumor cells via different mechanisms, such as granule exocytosis, plasmamembrane channels, or lysis (Fig. 1). Whichever mechanism is used, ATP release might play an unsuspected role in the therapeutic efficacy of anticancer agents. It is known that some therapeutics, such as mitoxantrone and oxaliplatin, owe their anticancer effect to the ability to trigger a strong antitumor immune response (immunochemotherapy; ref. 27). Very recent data show that chemotherapy-induced enhancement of antitumor immunity is largely mediated by ATP release from tumor cells (21, 28). In the extracellular space, ATP activates the P2X7 receptor of dendritic cells (DC), thereby triggering IL-1 β release, which in turn enhances Ag presentation to CD4⁺ T lymphocytes, and finally potentiates antitumor immunity. Thus, sensitivity to chemotherapy might depend on the ability of different tumors to release ATP. ATP release correlates with, and might even be dependent on, autophagy, an evolutionary conserved cell response to stressful environmental conditions, needed to induce an efficient chemotherapy-induced antitumor immunity (26). In response to mitoxantrone or oxaliplatin, autophagy-competent tumors release more ATP than autophagy-defective tumors, attract more DCs and T lymphocytes, and are more sensitive to chemotherapy. Interestingly, the sensitivity

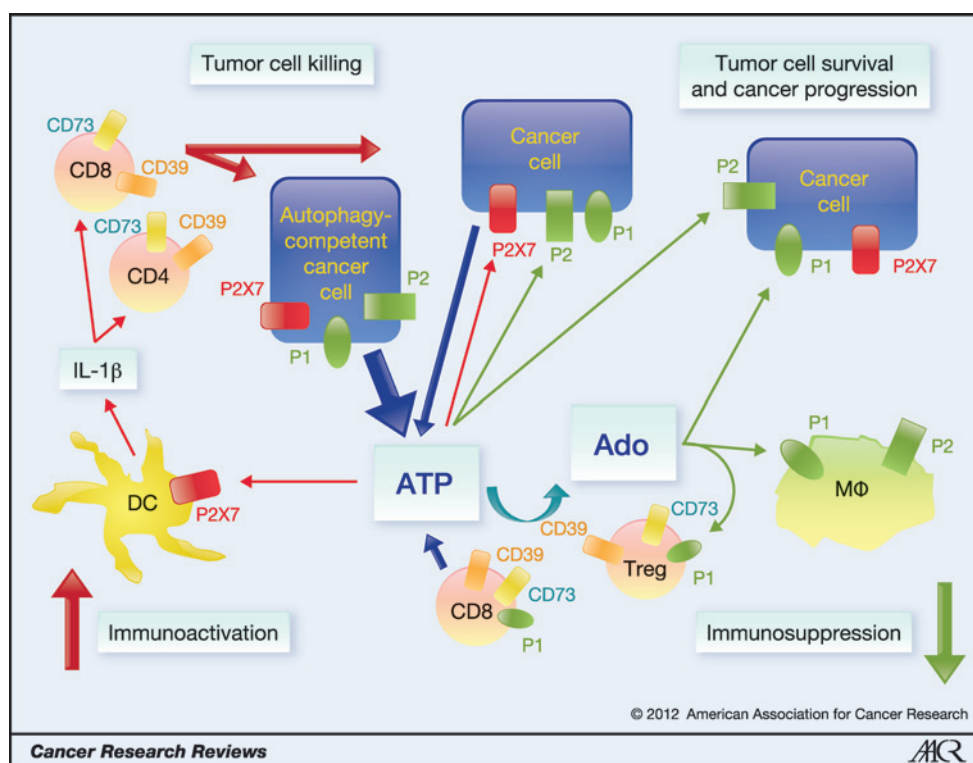


Figure 1. Modulation of immune response and cancer cell growth by ATP and adenosine in the tumor microenvironment. ATP is released by cancer cells and by infiltrating immune cells (thin blue arrows). A main source of extracellular ATP (thick blue arrow) are autophagy-competent cancer cells. Thanks to the sequential activity of CD39 and CD73 expressed on CD4⁺, CD8⁺, and Treg lymphocytes, ATP is degraded to adenosine (Ado). ATP acts at P2 receptors expressed on cancer and immune cells. Depending on the concentration and other poorly known factors, ATP may cause cancer cell proliferation (green arrows) or even cancer cell death (red arrow). P2X7 has an intriguing role, because its activation may cause proliferation as well as cytotoxicity. On the other hand, ATP drives immune cell recruitment and activation, and immuno-mediated cancer cell death. Of particular relevance is activation of DC P2X7, as this causes IL-1 β release and stimulation of T-cell-mediated anticancer responses (red arrows). Adenosine acts at P1 receptors, expressed on immune and cancer cells, causing immunosuppression and stimulation of cancer cell survival and proliferation (green arrows). Pathways in red denote antitumor responses; pathways in green denote protumor responses; blue arrows indicate ATP release.

to chemotherapy of autophagy-defective tumors can be restored by ecto-ATPase inhibitors (26). These data provide a clean demonstration of the crucial function of ATP as an immunomodulator in the tumor microenvironment (27, 28).

Extracellular Purines and Antitumor Immunity

The role of ATP in the complex dynamics of the host-tumor interaction is not always beneficial for the host. The final outcome depends on 2 crucial factors: (i) the panel of P2 and P1 receptors expressed on the tumor and infiltrating inflammatory cells, and (ii) the level of expression of nucleotide-hydrolyzing enzymes (CD39 and CD73). Depending on the local concentration and the P2 receptor subtypes expressed by the immune cells, ATP can act as an immunostimulant or an immunosuppressive agent. Acting at P2Y11, ATP may induce a semimaturational state of DCs, whereby release of proinflammatory cytokines (e.g., IL-1 β , TNF α , IL-6, IL-12) is downmodulated, whereas release of suppressive cytokines (e.g., IL-10 and IL-1Ra) is unaffected or enhanced (29). This remodeling of cytokine secretion results in an impairment of Th1 immunity and in a poten-

tiation of Th2-mediated responses and, thus, in a preferential activation of immunosuppressive rather than immunostimulatory pathways. In addition and more importantly, sequential ATP hydrolysis via CD39 and CD73 generates the potent immunosuppressive factor adenosine.

Adenosine is one of the most abundant biochemical constituents of the tumor microenvironment (30, 31), and for several years, it has been implicated in tumor-associated immunosuppression. However, unequivocal proof of its role has been produced only during the last 2 years. Originally, Blay and Hoskin, who postulated that adenosine was the factor responsible for immunosuppression within the tumor milieu, measured actual adenosine levels in microdialysates from the interstitial fluid of solid tumors and showed that adenosine inhibited tumor-infiltrating cytotoxic T cells (30, 32, 33). These observations were confirmed and further extended by Sitkovsky and coworkers who identified the P1 receptor subtype involved as A2A (31). More recent studies highlighted the crucial role in tumor progression of extracellular adenosine-generating systems.

The tumor microenvironment is hypoxic, a metabolic condition that induces accumulation of adenosine as a

consequence of accelerated hydrolysis of extracellular ATP. The main enzymes responsible are the CD39 and CD73 ectonucleotidases, which are upregulated by the hypoxia-induced transcription factors Sp1 and HIF-1, respectively (34, 35). Expression of E-NTPDase1 (CD39) or E-NTPDase2 by vascular endothelium or by tumor cells accelerates tumor growth and metastasis of rat glioma, mouse colorectal cancer, and mouse melanoma (36, 37), whereas on the contrary, CD39 gene deletion causes reduced growth of transplanted tumors in mice (38). Stagg and coworkers have shown in a series of elegant studies that AMP hydrolysis by CD73, the final step in the chain of reaction leading to adenosine generation, is crucial for tumor progression (39–41) because neutralization or deletion of CD73 inhibits tumor growth and prevents metastasis, supporting a major role for adenosine as a key component of the tumor-suppressive microenvironment. Importantly, these authors confirmed the earlier identification by Blay and Hoskin that CD8⁺ T lymphocytes are the main target of the immunosuppressive effect of adenosine (33, 41). Although CD8⁺ lymphocytes are the main target of adenosine, the main source of this nucleoside are tumor-infiltrating T-regulatory cells (Treg), which express to a high level both CD39 and CD73 (42–44). These ectonucleotidases are thought to be largely responsible for the immunosuppressive activity of Tregs.

Among the main T-lymphocyte subsets, T_H17 cells are emerging as important determinants of antitumor response that promote tumor growth or regression, depending on poorly understood host or tumor factors. In a recent article, Ghiringhelli and coworkers showed that tumor-infiltrating T_H17 lymphocytes express CD39 and CD73 and are thus able to suppress T-effector cell functions via adenosine generation (45). This observation suggests that the different effect of T_H17 lymphocytes on tumor fate might depend on their level of ectonucleotidase expression (45). Interplay between purinergic signaling and T_H17 cells functions is complex and to date poorly understood. See, for example, a recent study showing that ATP may also inhibit suppressive activity of Tregs and drive their differentiation into T_H17 lymphocytes (16). How this might affect host–tumor interaction is unknown.

In the extracellular space, adenosine is degraded to inosine by adenosine deaminase (ADA). Activity of this enzyme is strongly dependent on its interaction with the cell-surface serine protease dipeptidyl peptidase IV/CD26, which acts as an ADA-binding protein. It is anticipated that enhanced degradation of adenosine, and thus enhanced expression of CD26, should negatively correlate with tumor growth. However, literature data are controversial; in some tumors (e.g., melanoma, ovarian carcinoma, or non–small cell lung carcinoma), lack of CD26 correlates with an aggressive behavior, whereas in other cases, CD26 presence is associated with faster tumor progression (e.g., prostate cancer, mesothelioma, or thyroid carcinoma; ref. 46). It is likely that these contrasting effects are due to the multifunctional role of CD26, a glycoprotein that associates with several different plasma membrane or extracellular proteins (e.g., fibroblast-

activating protein- α , plasminogen, CXCR4, CD45) besides ADA. Moreover, CD26, by cleaving *N*-terminal dipeptides from polypeptides with proline or alanine in the penultimate position, can modulate activity of many cytokines and chemokines.

Purinergic Receptors Modulate Tumor Cell Growth

Most studies have so far concentrated on the effect of purinergic signaling on tumor-associated immune response; however, nucleotides and nucleosides have strong direct effects on the tumor cells themselves. Stimulation of P2Y receptors (e.g., P2Y1 and P2Y2) supports growth, thus it is likely that, depending on the P2Y receptor subtypes expressed, accumulation of ATP into the tumor microenvironment promotes tumor growth. In addition to P2Y receptors, P2X7 is involved in tumor growth. It is a long-standing observation that most malignant tumors overexpress P2X7 (47). This is somewhat surprising because this receptor is known to mediate a strong cytotoxic response (48); thus, one wonders why tumors should overexpress a "suicide" receptor. However, cytotoxicity is most commonly triggered by pharmacologic (i.e., near millimolar) ATP doses. On the contrary, P2X7 activation by endogenously released ATP produces a trophic, growth-promoting effect (49, 50). Accordingly, tumors engineered to overexpress P2X7 show an accelerated *in vivo* growth rate, higher VEGF release, thicker vascular network, and increased tendency to metastasize (51, 52). Increased metastatic activity of P2X7-expressing cells is of particular relevance in the context of the oncogenic activity of this receptor. Enhanced invasiveness conferred by P2X7 expression is likely to depend not only on VEGF release but also on the ability to release proteases (52, 53). On the other hand, P2X7 silencing or pharmacologic blockade slows down tumor progression (51). P2X7 activity depends on the presence of spontaneously released ATP in the tumor milieu, as shown by the observation that coinjection of apyrase, together with tumor cells, inhibits tumor growth (54).

Adenosine directly affects tumor cell growth, mainly via the A3 receptor, which is upregulated by different tumors (7). Stimulation of A3 receptors is reported to have growth-promoting as well as growth-inhibitory effects, antiapoptotic or proapoptotic effects. Possible implication of A3 receptors in cancer growth has prompted several *in vivo* studies based on the administration of A3 agonists to take advantage of the A3 proapoptotic activity. Initial results are encouraging in several tumor models (e.g., PC3 human prostate carcinoma, HCT-116 human colon carcinoma, CT26 mouse colon carcinoma, hepatocellular carcinoma; refs. 7, 55).

Purinergic Signaling and Cancer Therapy

Convergent reports documenting the activity of ATP and its metabolites on cancer growth *in vivo* raise the obvious issue of how we can take advantage of purinergic signaling to defeat cancer. Schematically, two avenues are possible for intervention on the host and/or on the tumor side. Available

evidence in several experimental tumor models clearly shows that reducing the adenosine concentration halts tumor progression and prevents metastasis (40). Adenosine concentration in the tumor interstitium can be lowered by downregulating CD39 and/or CD73, or by upregulating CD26. Alternatively, ADA could be targeted to the tumor as a PEG-ADA conjugate (56). Again, on the host side, the beneficial effect of ATP release is shown by its ability to activate the P2X7/inflammasome axis and recruit immune cells into the tumor (21, 26). A pharmacologic strategy might thus be based on the administration of CD39 inhibitors, with the dual beneficial effect to preserve ATP levels sufficient for immunostimulation and prevent adenosine accumulation.

On the tumor side, the strategy for a possible therapeutic intervention is much less clear. Overexpression of P2X7 by the tumor might be exploited to kill the tumor cells; thus, administration of a selective P2X7 agonist might be a therapeutic option (57). This approach exploits the well-known proapoptotic effect exerted by pharmacologic P2X7 overactivation (58). It is known that in some epithelia, baseline apoptosis depends on P2X7 activation. Gorodeski and coworkers have taken advantage of this proapoptotic effect to antagonize the growth of chemically induced papillomas in mice (59). However, given the growth-promoting activity of P2X7, the opposite strategy is also feasible: administration of P2X7 blockers to inhibit tumor growth and metastasis (60). *In vivo* experiments show that both options are worth pursuing.

A few studies have explored the therapeutic activity of ATP infusion in patients with cancer (61, 62). The treatment is well tolerated, and effects on nutritional status, overall quality of life, and long-term survival are encouraging, although the mechanism of action of this systemic ATP administration is not clear. Finally, adenosine is also active on tumor cells (7). However, it is not yet clear whether administration of P1

agonists or antagonists will be beneficial or detrimental, due to the different P1 subtypes expressed and the different dose-dependent effects on proliferation, VEGF release, and apoptosis; current evidence suggests that targeting A3 receptors might be a promising anticancer strategy (63).

Conclusion

Nucleotides and their receptors are emerging as novel and important modulators of inflammation and immunity and, as such, potential players in host–tumor interaction. Given the large number of P2 and P1 receptors expressed by tumor and inflammatory cells, the widely different ligand affinity of the different P2 and P1 receptor subtypes, the modulation of receptor expression by local factors, and the effect of ectonucleotidases and ADA on the nucleotide and adenosine concentration, predicting the final result on tumor progression and metastasis formation of purinergic signaling interference is very difficult. However, *in vivo* data support *in vitro* evidence that lowering the intratumour adenosine concentration and targeting the P2X7 receptor have a strong antitumor effect. Thus, investigation of purinergic signaling in cancer opens promising perspectives for the development of innovative therapeutics.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

F. Di Virgilio is supported by grants from the Italian Association for Cancer Research (IG 5354), Telethon of Italy (GGP06070), the Ministry of Education (FIRB RBAP11FXBC and PRIN 2009LMEEEH), the European Community (ERANET Nanostroke), and institutional funds from the University of Ferrara.

Received April 23, 2012; revised May 21, 2012; accepted May 30, 2012; published OnlineFirst October 22, 2012.

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