Mesenchymal stem cells: building blocks for molecular medicine in the 21st century

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Mesenchymal stem sells (MSCs) are present in a variety of tissues during human development, and in adults they are prevalent in bone marrow. From that readily available source, MSCs can be isolated, expanded in culture, and stimulated to differentiate into bone, cartilage, muscle, marrow stroma, tendon, fat and a variety of other connective tissues. Because large numbers of MSCs can be generated in culture, tissue-engineered constructs principally composed of these cells could be re-introduced into the *in vivo* setting. This approach is now being explored to regenerate tissues that the body cannot naturally repair or regenerate when challenged. Moreover, MSCs can be transduced with retroviral and other vectors and are, thus, potential candidates to deliver somatic gene therapies for local or systemic pathologies. Untapped applications include both diagnostic and prognostic uses of MSCs and their descendents in healthcare management. Finally, by understanding the complex, multistep and multifactorial differentiation pathway from MSC to functional tissues, it might be possible to manipulate MSCs directly *in vivo* to cue the formation of elaborate, composite tissues *in situ*.

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The body houses several types of progenitor cells that are capable of dividing many times, while also giving rise to daughter cells with more restricted developmental potentials. Eventually these cells differentiate and have specific phenotypic characteristics that contribute to their highly specialized function. Examples of such stem cells include, of course, the totipotent zygote, as well as embryonic stem cells (ESCs) (Ref. 1), hematopoietic stem cells (HSCs) (Ref. 2) and mesenchymal stem cells (MSCs) (Refs 3,4). Within each category of stem cell – and this list is by no means all-encompassing – the constituents can be broadly distributed throughout the body and capable of differentiating along very specific lineage pathways to unique differentiated phenotypes. In the case of HSCs, it is now commonly understood that all of the cellular elements of the blood can be derived both *in vivo* and *in vitro* from a purified population of cells possessing the CD34 antigen on their surface[5].

In the case of MSCs, the lineage committed cells can fabricate a spectrum of specialized mesenchymal tissues including bone, cartilage, muscle, marrow stroma, tendon, ligament, fat (Fig. 1) and a variety of other connective tissues[6]. This class of progenitors, the MSCs, resides in bone marrow, around blood vessels (as pericytes), in fat, skin, muscle and other locations. Although antibodies to several cell surface antigens can be used to recognize MSCs (Refs 7–10), monospecific and unique molecular probes do not exist to unequivocally identify these cells *in situ*; as such, it is currently difficult to quantify their actual numbers or identify their precise locations. Nevertheless, there is a growing body of literature to indicate that the number of MSCs decreases with age or infirmity[11–16], and that their relative presence can control the outcome of reparative events of skeletal tissues. In sum, the complement, or titer, and performance of MSCs can have a dramatic impact on the overall health status of individuals by controlling the body's capacity to naturally remodel, repair, and upon demand, rejuvenate various tissues.

Within the overall context of developmental and stem cell biology, the concept of plasticity has drawn considerable attention of late[17]. Until recently, it was believed that tissue-specific, or at least germ layer-specific, stem cells gave rise to mature differentiated phenotypes only within their restricted downstream lineages. Several lines of evidence now challenge the notion that such limitations exist, as HSCs were proven to form hepatocytes[18], muscle satellite cells demonstrated to form hematopoietic elements[19] and MSCs shown to form glial populations[20]. Within the mesenchymal cell lineages, plasticity of mature cells, not stem cells, was proposed over twenty years ago by those showing that highly differentiated chondrocytes could 'transdifferentiate' into osteoblasts[21], and later by Bennett *et al.*[22], who showed that adipocytes could switch their phenotype to that of osteoblasts as well. Despite the valuable perspectives gained from detailed studies of HSCs, recently put forth by Weissman[23], the precise relationship between mature and progenitor cells of the marrow stromal compartment remains unclear at both the phenomenologic and mechanistic levels[24], and might, in fact, bear little resemblance to the HSC paradigm. This review will not address the complexities of regulating mesenchymal tissue formation and the possible plasticity of MSCs into heterologous tissue lineages, but rather, aims to outline some of the clinical opportunities the future holds now that we have the ability to isolate and manipulate MSCs.

The state of the art

In the late 1980s, the technique for isolating, purifying and mitotically expanding MSCs from marrow specimens of individuals of any age was optimized[25].

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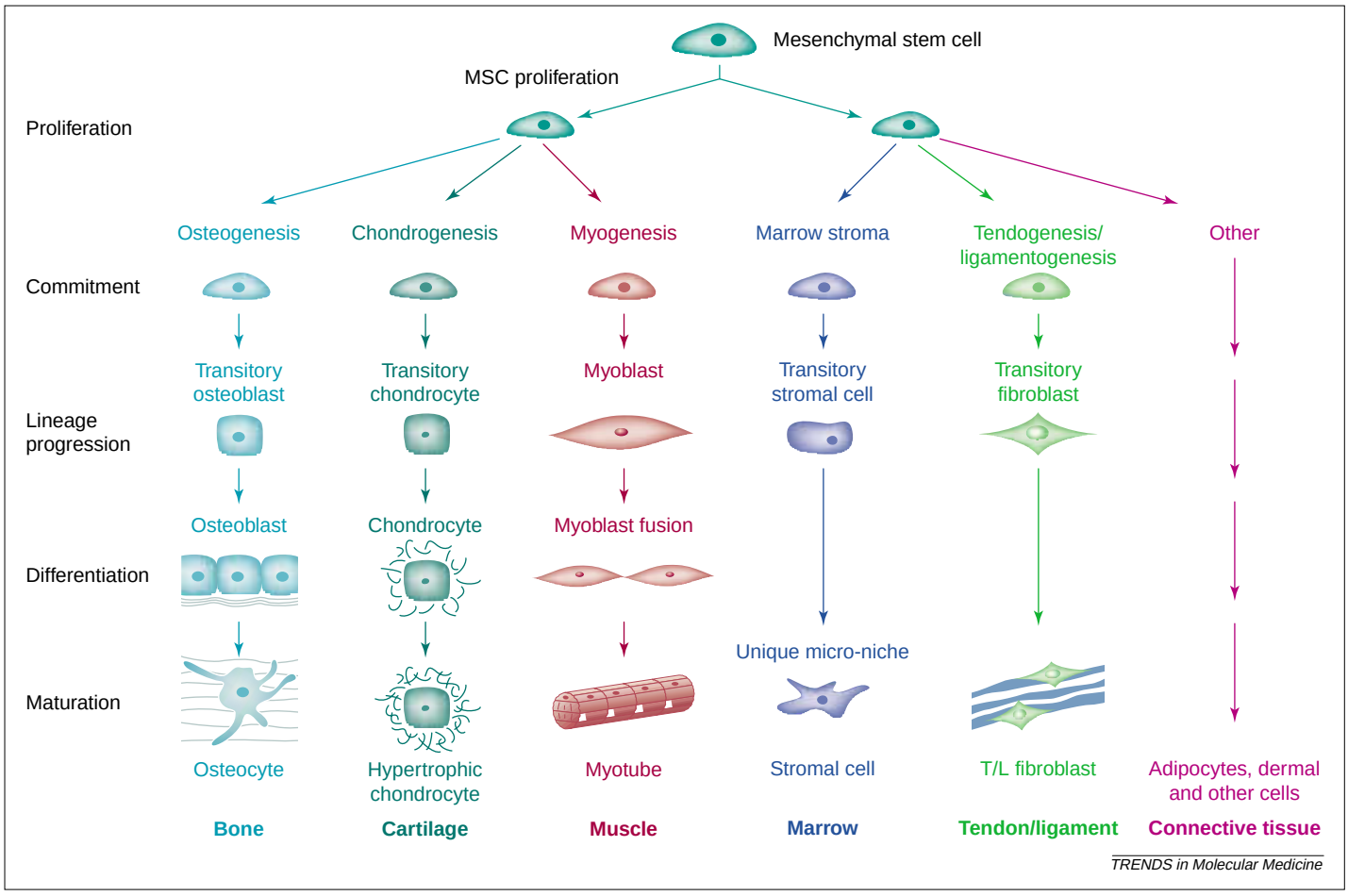

Fig. 1. The mesengenic process. The stepwise cellular transitions from the putative mesenchymal stem cell (MSC) to highly differentiated phenotypes are depicted schematically. This scheme is oversimplified and does not represent all of the transitions or all of the complexities of single lineage pathways, nor does it represent the potential interrelationships of cells moving between pathways, now commonly referred to as 'plasticity'. The individual lineage pathways are arranged from left (best understood) to right (least understood); the osteogenic[54] and chondrogenic[55] pathways are based on detailed experimental information. It is believed that major mitotic expansion takes place in marrow/periosteum or at sites of massive mesenchymal tissue repair, and the highly differentiated cells possess a substantially restricted proliferative potential. For additional information about the molecular control of each lineage pathway, see Refs 56–61.

With an expansion potential exceeding one billion-fold in culture[26], and the subsequent availability of large numbers of MSCs, studies were initiated to further characterize these cells and their response to various bioactive molecules. Although inductive agents that cause the entrance into and progression along individual lineage pathways for bone[27], cartilage[28], fat[4] and muscle[29] are known, the molecular details that govern regulation of each lineage pathway continue to be active areas of investigation. It is also quite clear that MSCs constitutively secrete specific growth factors and cytokines[30] and that induction into each differentiation pathway involves modulation of the synthesis of these secreted molecules and the regulation of other proteins in a lineage path and stage-specific manner[31]. Importantly, each preparation of MSCs has donor-specific levels of constitutively secreted cytokines, even though the percentage of up- or downregulation by inductive agents is comparable[30]. This observation allows us to introduce the notion of a personalized biological 'set point' for bioactive factor elaboration and homeostasis, against which perturbations in the relative level of selected bioactive factors can be deduced or measured in various states of health and disease. Taken a step further, we propose that the genotype of each individual controls the secretion of, and responsiveness to, cytokines and growth factors produced by MSCs or other cells in culture and, presumably, *in vivo*. How this affects prognosis, diagnosis and treatment of mesenchymal tissue dysfunction will be discussed later in this article.

This hypothesis regarding biological 'set points' begs the question of individual responsiveness to pharmacological and other therapeutic interventions in the practice of clinical medicine. It is extremely interesting, and somewhat perplexing, that standard doses of most pharmaceuticals produce the desired effect across wide population spectra. This would not necessarily be expected in light of the observation that the basal level of numerous growth factors and the cellular responsiveness varies from person to person. Rather, it would seem that, in view of the quantitative differences in intermediate cellular signaling cascades, individualized therapeutic regimens would be more efficient and effective. Later, we will propose some recommendations on how the study of MSCs might guide customized treatment plans for patients in the 21st century.

Developing suitable animal models to study the engineering of MSCs to effect the regeneration of skeletal tissues is a complex issue. Not only are there subtle differences in the sensitivity and behavior of the biological elements such as MSCs, but the mechanical loads, environment and architecture of skeletal components vary widely across species. For example, dexamethasome induces human MSCs *in vitro* into the osteogenic lineage[27], yet it induces mouse MSCs into the adipocyte lineage[32]. Conversely, recombinant human bone morphogenetic protein 2 (rhBMP-2) in low doses induces mouse MSCs into the osteogenic lineage[33], but very high doses are required to see the same effect on human MSCs (Ref. 34). Thus, the responsiveness of MSCs to certain powerful inductive molecules can be quite different depending on the animal. However, the downstream molecular details of osteo-induction are similar in many aspects including the observation that both mouse and human cells require the presence of the transcription factor Cbfa1 for osteoblastic differentiation[35]. Although many generalities regarding the behavior of MSCs are conserved across the phylogenetic tree, great care must be exercised in extrapolating the details gleaned from animal MSCs to the case of human MSCs.

Given that large numbers of autologous MSCs can be generated in cell culture, they represent an essential ingredient required for successful bioengineering of new human tissue. To recreate functional tissues, the key ingredients are extracellular scaffolds to anchor, deliver and orient cells, bioactive factors to provide the instructional and molecular cues, and cells capable of responding to their environment by synthesizing the new tissue of interest. Given the multi-lineage potential of MSCs, their exquisite sensitivity to specific signaling molecules, and their relative ease of handling *in vitro*, they are a potentially powerful tool in tissue engineering. This approach has been employed, using animal models, for bone[36], cartilage[37], tendon[38], marrow stroma[39] and muscle[40] repair and regeneration.

Both animal and human MSCs in culture have been successfully transduced with exogenous genes using several different vectors without an apparent defect of their stem cell properties[39,41]. This allows the tracking of genetically marked MSCs *in vivo*, and the introduction of normal genes into animals with dysfunctional mutations. An example of such gene therapy is the correction of osteogenesis imperfecta by the introduction of MSCs with a wild-type gene for type I collagen[42–44]. In addition, MSCs have been transfected with genes for growth factors and cytokines, and shown to express the proteins *in vitro* through multiple rounds of stable cell division[39,45]. As a further example of their potential use, Lieberman and colleagues[46] transfected animal MSCs with the gene encoding rhBMP-2, locally implanted the cells back at the site of a large bone defect of the femur, and observed substantial healing within 8 weeks. These studies confirmed that MSCs could be used to deliver a clinically relevant growth factor, and that a portion of the implanted cells themselves were competent to respond to the factor in an autocrine or paracrine fashion. In such a genetic engineering context, congenic but normal mouse MSCs (allograft) have been used to correct a specific gene defect by injecting the 'normal' MSCs into a specific muscle of the *mdx* mouse[40] – a murine model of muscular dystrophy. The formation of new, normal muscle locally demonstrates that MSCs have myogenic potential, and that delivery of the normal dystrophin gene in the syngeneic MSCs implanted into *mdx*-muscle can 'cure' the defect.

Future trends

Tissue protection, repair and replacement

The surgical reconstruction of any tissue will require reparative cells and the appropriate scaffold to both introduce the cells into the wound site and support tissue specific biosynthetic events. Additionally, inductive and phenotype-specific growth factors and cytokines must sequentially interact with the implanted cells and their progeny to effectively consummate a tissue engineered reformation of functional tissue. Hybrid tissues, such as articular cartilage on a bed of subchondral bone, or tendinous tissue with a functional osseous anchor, represent additional challenges for MSC-based constructs. Eventually, the *ex vivo* formation of complex three dimensional tissues with integrated vascular access for implantation would revolutionize the treatment of damaged or diseased tissue. The conceptual basis for this expectation is the current use of allograft composite tissues to replace diseased or dysfunctional skeletal tissue such as the condyle of the knee[47].

Once we have the ability to control the formation of repair tissues *in vitro* or *in vivo*, the next frontier for providing clinical advantage lies in the prefabrication of tissues that can be created without the lengthy and onerous process of stem cell harvest, purification, culture expansion and process validation. Preliminary experiments now suggest that both human and animal MSCs do not express co-stimulatory antigens such as B7 and, as such, appear to be immunoprivileged. In a recent canine study, major histocompatibility complex (MHC)-mismatched MSCs implanted in large osseous defects were capable of forming a substantial amount of new bone in the wound site without evidence of immunologic rejection[48]. Although the molecular mechanisms underlying this interesting observation have not been identified, these results point to the possibility of establishing universal donor cells for such tissue engineering applications. In essence, these cells might only be necessary to initiate the tissue regeneration cascade; integration of the new or remodeled tissue could result from the subsequent contribution of mobilized, autologous MSCs to the repair site.

In human clinical research, initial efforts are focused on applications of MSC-based tissue repair that do not require a three dimensional scaffold for cell

delivery. The reasons for this are many, but principally owing to the governmental regulatory hurdles associated with combining cells and implantable devices, and the lack of biomaterials approved by the FDA that are suitable for structural tissue regeneration. Because MSCs can differentiate into the specialized marrow stroma that provides structural and instructional support for hematopoiesis, MSCs have been used as an adjuvant to the infusion regimen of bone marrow transplantation following high dose chemotherapy[10,49,50]. The hypothesis was that, following ablation or injury of the native marrow stroma and infusion into the vascular network, MSCs would selectively 'home' back to the bone marrow from which they originated to re-fabricate a supportive marrow stroma. The efficiency of homing was never measured but data available from recent studies[51] indicate that it is quite low, perhaps in the range of 1–2% of the infused cells. Increasing the efficiency of MSC engraftment and targeting the infused cells to specific tissue locations could have a large impact on future therapeutic uses of MSCs for other diseases. The most obvious candidate for systemic enhancement of an individual's MSC titer is probably post-menopausal or age-related osteoporosis, as a diminished bone-forming capacity might be caused by a reduced cache of local MSCs[11,13–15]. Additionally, MSCs could be injected into diarthrodial joint spaces to contribute to repair or regeneration of meniscal or articular cartilage[52]. Methods to stimulate expression of specific cell surface ligands, either natural or engineered, that can mediate selective attachment to known receptors in target tissues of interest will be of considerable clinical benefit.

With the development of implant materials that encapsulate a variety of growth factors and cytokines, it should someday be possible to manipulate endogenous MSCs to promote tissue regeneration. This would involve the sequential chemoattraction of MSCs, their site-specific mitotic expansion, their induction into and through a specific phenotypic differentiation pathway, the integration of neo-tissue with host tissue and the modulation thereof to match the tissue site. Currently, the site-specific local delivery of just a single bioactive factor to act upon MSCs remains a clinical challenge. Clearly, the *in vivo* management of MSCs will require a more detailed understanding of the cellular and molecular players of each differentiation pathway. Of course, co-management of other tissues, such as the vascular system, will probably be obligatory for development and fabrication of elaborate structures such as bone. Whether the 'bioactive factors' that are used to stimulate these processes are soluble recombinant molecules that bind to traditional cell surface receptors and initiate a classical signal transduction pathway remains to be determined. Alternative approaches for modulating the endogenous sequence of intracellular events in MSCs could be aimed at developing small, agonistic or antagonistic synthetic molecules with specific cytoplasmic targets, or developing novel compounds capable of crossing the nuclear membrane and stimulating transcriptional events at the promoter level of the genome. Finally, for pathologic conditions where mesenchymal cell growth and/or differentiation goes unchecked, such as various tumors or the devastating heterotopic ossification disease known as fibrodysplasia ossificans progressiva[53], repression of MSC proliferation and lineage progression could be specifically controlled by these proposed designer molecules at the level of individual cells or clusters.

Prognostic and diagnostic use of MSCs

The Human Genome Project will eventually identify all human genes. It will also allow the identification of dysfunctional mutations. It will not, however, be able to immediately assist in defining the expressional profiles and the quantity or activity of translated molecules for each individual. Indeed, human health care is severely limited by its inability to have accurate phenotypic descriptions of each patient and how these attributes change with age or disease states. As noted above, the unique genotypes of the human population would predict quite different drug and bioactive factor reactivities of each patient. Serum chemistries, blood counts, cutaneous characteristics, pain thresholds and physical palpation are currently used to phenotype patients as part of a routine examination. With the ability to isolate, expand and study MSCs *in vitro*, individual patient's MSCs could be tested for their sensitivity to various drugs, cytokines, growth factors and inductive agents. In this setting particularly, where treatment would depend on bioactive factor or cytokine therapy, we propose the creation of individual dosing regimens based on the *in vitro* responsiveness in a simple assay performed using a patient's own MSCs. Optimized treatment plans could then be created that efficiently and precisely integrate with the host's expected biological response. For example, parathyroid hormone (PTH) is currently being used to systemically influence the anabolic activity of bone-forming cells. A patient's sensitivity to a specific dose range of PTH would be determined in cultures of his MSCs that are induced into the osteogenic lineage pathway. Likewise, if accurate assays of MSC titers could be devised, both the responsiveness to stimulation and the number of responding cells should be assayed, and this information used for therapeutic management of individual patients suffering from one of several bone diseases. In this way, the genetic repertoire of each patient's MSCs direct a customized treatment plan in the forthcoming age of molecular medicine.

Because all mesenchymal tissues turn over and rejuvenate themselves, MSCs must be involved in this constant rejuvenation process. By understanding how this turnover activity is controlled and how it changes

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with the patient's age, both prognostic and diagnostic assays can be established to both predict and, then, treat a specific patient. It could be that if we can accurately predict that a patient will be at risk for a mesenchymal tissue dysfunction (e.g. osteoporosis, osteoarthritis, collagen-vascular diseases) in 10 or 20 years time, early management of MSCs could be initiated to thwart the predicted dysfunction. Rather than observing that a grandmother has osteoporosis and, thus, her daughter will also have this disease, we should be able to assay both the quantity and osteogenic potential of the MSCs and determine how to increase the bone-forming capacity of the daughter. In this way, we will aim to modulate the bone-forming rate at the molecular level to preclude catastrophic bone loss. The specific targets for these medical applications will require a more detailed understanding of the mesengenic pathway (Fig. 1) and its molecular management.

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Presenilins as therapeutic targets for the treatment of Alzheimer's disease

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Studies demonstrating that accumulation and aggregation of the amyloid β protein (Aβ) within the brain is likely to cause Alzheimer's disease (AD) have provided the rationale for therapeutic strategies aimed at influencing Aβ production, aggregation and clearance. γ-secretase catalyzes the final cleavage that releases the Aβ from its precursor; therefore, it is a potential therapeutic target for the treatment of AD. Recent data show that the polytopic membrane proteins presenilin 1 and presenilin 2 are either catalytic components or essential co-factors of a membrane-bound proteolytic complex that possesses γ-secretase activity. Although recent findings demonstrating that γ-secretase inhibitors bind directly to presenilins (PSs) further support a catalytic role for PSs in γ-secretase cleavage, additional studies are still needed to clarify the role of PSs in γ-secretase cleavage and the use of targeting PSs to reduce Aβ production.

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One of the pathological hallmarks of Alzheimer's disease (AD) is the deposition of the ~4 kDa AMYLOID β PROTEIN (Aβ) (see Glossary) within lesions known as senile plaques. Aβ is also deposited in the walls of cerebral blood vessels in many cases of AD. Substantial proportions of the Aβ that accumulates in the AD brain is deposited as AMYLOID. Amyloid is, by definition, highly insoluble, proteinaceous material with a β-pleated-sheet conformation that is deposited extracellularly in the form of 5–10 nm wide straight fibrils. In the 17 years since the biochemical purification of Aβ from the amyloid deposits in AD brain, a great deal of evidence has emerged, which indicates that AD is caused by Aβ aggregation[1]. As Aβ aggregation is a concentration dependent phenomena, lowering Aβ levels might slow or prevent the development of AD. Thus, inhibiting Aβ production is likely to be therapeutic, and the proteases that produce Aβ, as well as the factors that regulate their activity, are major targets for drug discovery. Aβ is produced from the amyloid β protein precursor (APP) through two sequential cleavages. APP is first cleaved by β-SECRETASE at the N-terminus of Aβ to generate a large secreted derivative (sAPPβ) and a membrane bound APP C-terminal fragment (CTFβ). Subsequent cleavage of CTF by γ-secretase results in the production of Aβ peptides of varying length with the two species of most interest being a 40 amino-acid Aβ peptide (Aβ40)and a 42 amino-acid Aβ peptide (Aβ42), along with the cognate CTFγ. In an alternative, presumably non-pathogenic pathway, APP is cleaved within the Aβ sequence by α-secretase, which generates another large secreted derivative (sAPPα) and CTFα. Like CTFβ, CTFα can be cleaved by γ-secretase-yielding P3 and CTFγ (reviewed in Ref. 1) (Fig. 1). Significantly, the ability to monitor both Aβ production and other APP derivatives from cultured cells preceded the more definitive characterization of the β- and γ-secretase activities by many years. Thus, Aβ-lowering agents could be identified in cell-based screens and then categorized based on their effects on APP processing without definitive knowledge of their molecular target (Table 1). At this time at least one such compound (an inhibitor of γ-secretase activity) is currently in phase I clinical trials.

β-secretase is a novel aspartyl protease

Recently, a pepstatin-insensitive, transmembrane aspartyl protease (BACE, Asp-2) has been identified as the protease responsible for β-secretase cleavage[2–6]. Although development of β-secretase inhibitors has not been as widely reported as γ-secretase inhibitors (presumably because cell-based screens did not reveal many inhibitors of β-secretase cleavage), the identification of β-secretase as a member of the aspartyl protease family should greatly speed the development of such inhibitors. Indeed, within a year of its identification the initial

 1471-4914/01/$ – see front matter PII: S1471-4914(01)01991-8