however, that some controversial observations and/or interpretations, probably due to their complicated genetic backgrounds, have also been described. Because the SAM strains are polygenic, those specific genetic factors accounting for the observed bone phenotypes are still awaiting elucidation.

2. Premature aging syndromes in genetically modified mice

As described above, aged animals appear to be good models for senile osteoporosis. However, the mechanisms underlying the aging process are complex, making it difficult to decipher at the molecular level. Using knockout or transgenic techniques, some of these genetically modified mice result in premature aging phenotypes. The significant conclusion to be taken from these studies is that single gene mutations cause multiple aging phenotypes. This advantage is useful in the search for clues to the regulation of bone metabolism during the aging process (Hishiya and Watanabe, 2004).

Mouse models for human progeroid syndromes have also been reported (Kuro-o, 2001; Hasty et al., 2003; Warner and Sierra, 2003; Hasty and Vijg, 2004; Kipling et al., 2004). Genetically modified mice, which exhibit multiple aging phenotypes and shortened life span, are listed in Table 1. Werner syndrome is caused by a loss-of-function mutation in *WRN*, which encodes the RecQ family DNA helicase, and plays a role in genome stability, including telomere maintenance (Yu et al., 1996). Unexpectedly, knockout mice for the *Wrn* gene are essentially normal and exhibit no characteristics of premature aging (Lombard et al., 2000). These mice have long telomeres and relatively high telomerase activity, suggesting that the aging phenotype is latent as a result of residual telomere maintenance activity. Evidently, double knockout mice for *Wrn* and *Terc*, which encode the RNA component of telomerase activity, show a Werner-like phenotype with osteoporosis (Chang et al., 2004; Du et al., 2004). Recently, a gene encoding lamin A has been identified to be responsible for the human progeria, Hutchinson-Gilford syndrome (De Sandre-Giovannoli et al., 2003; Eriksson et al., 2003). Mice carrying an autosomal recessive point mutation in the lamin A gene, corresponding to the mutation identified in humans, also develops a progeria-like phenotype with osteoporotic symptoms (Mounkes et al., 2003).

Besides these authentic models for human premature aging syndromes, mice presenting with multiple aging phenotypes have also been reported. Null mutations of the Ku86 gene, also known as Ku80, which plays a role in DNA repair and transcription, exhibits shortened life span and elicits a premature aging phenotype, including osteopenia (Vogel et al., 1999). The aging phenotype has been also observed in mice lacking PASG, an SNF-like molecule that functions in DNA methylation (Sun et al., 2004). Mutant mice show decreased BMD and a secondary ossification delay in the tibial epiphyses (Sun et al., 2004). In addition to the genetic mutations involved in genomic stability and nuclear organization, mice carrying mitochondrial DNA polymerase mutations, resulting in loss of the region responsible for its proofreading activity, also show the osteoporotic phenotype along with

Table 1
Genetically modified mice exhibited premature aging phenotype and/or short life span

Gene name	Function	Modification	Bone phenotype	Characterization of bone
BubR1	Spindle assembly checkpoint	Hypomorph	Normal (kyphosis)	DXA
Klotho	Oxidative stress response, mineral metabolism?	Hypomorph	Osteoporosis	SXA, microCT, histological analysis, cell culture
Ku86	DNA repair, transcription	KO	Osteopenia	(not indicated)
MsrA	Oxidative stress response	KO	N/D	
mTR	Telomere maintenance	KO[a]	Normal	X-ray analysis, histological analysis
PASG	DNA methylation	Hypomorph	Osteopenia	X-ray analysis, histological analysis
PolgA	Mitochondrial DNA replication	Knock-in	Osteoporosis	X-ray analysis
Prdx1	Oxidative stress response	KO	N/D	
Top3b	DNA replication and repair	KO	N/D	
TRp53	Cell cycle	Deletion mutant	Osteoporosis	X-ray analysis, histological analysis
		Mutant Tg	Osteoporosis	X-ray analysis
		Short isoform Tg	Osteoporosis	X-ray analysis, histological analysis
XPD	DNA replication and repair	KO	Osteoporosis	X-ray analysis
Wrn/Terc	Telomere maintenance	Double KO	Osteoporosis?	microCT

N/D, not described. DXA, dual energy X-ray absorptiometry; SXA, single energy X-ray absorptiometry; microCT, microcomputed tomography.
[a] The phenotype was observed in the 6th generation from mTR knockout mouse matings.

other premature aging symptoms (Trifunovic et al., 2004); in particular, decreased bone mass, which is now known and appreciated as a hallmark of the premature aging phenotype. However, most observations of the skeletal phenotype were examined by X-ray analysis. The pathophysiology, including histology, of the bone phenotypes in these models for premature aging has not been well described, as yet.

Errors in cell duplication, such as those mis-programmed by the above-mentioned mutations, can be detected and corrected by arresting cell cycle (Fig. 1). Checkpoint signaling systems play critical roles in those programs (Hartwell, 1992; Nurse, 1997). Checkpoint kinase cascades are involved in DNA replication and other cell cycle events. ATM is a PI3K family kinase involved in DNA repair and oxidative response (Rotman and Shiloh, 1998). The gene encoding the kinase has been identified as a gene mutated in ataxia telangiectasia, recognized as one of the human premature aging syndromes (Lavin and Shiloh, 1997). Knockout mice for *Atm* exhibit a similar phenotype to the human disease, including hyperradiosensitivity and ataxic defects (Barlow et al., 1996; Elson et al., 1996; Xu et al., 1996). The osteopenic phenotype has also been observed in these mice. Differentiation of osteoblastogenesis and osteoclastogenesis, however, is rather normal. Colony formation assays revealed that the phenotype was the result of the proliferative defect in bone marrow mesenchymal

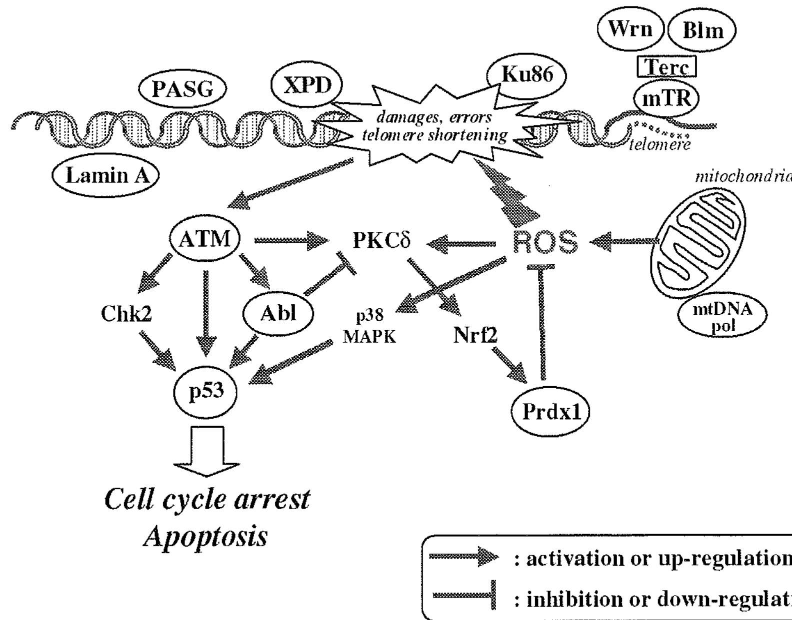

Fig. 1. Predicted pathways connecting the gene products responsible for the premature aging mutant phenotype. Most of the mouse models for premature aging are caused by mutations in the genes involved in genomic integrity and subsequent cell cycle regulation. Errors and damage to the genome or telomere shortening, which also affects DNA integrity could, in theory, be detected and corrected. Mutations in the genes responsible for genomic stability cause accumulation of phenotypic abnormalities. Genomic disorganization activates cell cycle-regulating pathways involving checkpoint kinases and p53. Oxidative stress is among the triggers that elicit genomic instability via DNA damage. Elevation and excess of ROS affect downstream signaling, including PKCδ, which subsequently stimulates the anti-ROS pathway, including transcriptional activation of Prdx1.

stem cells or progenitors (AH et al., submitted). The gain-of-function mutations in p53, a downstream effecter of ATM kinase, also result in a premature aging phenotype with osteoporosis (Tyner et al., 2002; Maier et al., 2004). Among them, p44 transgenic mice show a low turnover with a significant decrease in osteoblast number, along with a slight reduction of osteoclasts (Maier et al., 2004). Although further evaluation of each model is still required, these studies suggest that stem cell defects resulting from DNA damage and subsequent cell cycle arrest or other cell cycle abnormalities, at least in part, may account for the decreased bone formation and subsequent osteopenia observed in these premature aging models.

Other models for accelerated aging exist for which the responsible genes are apparently not directly involved in genomic integrity. For example, mice carrying hypomorphic mutations of the gene called Klotho show multiple aging phenotypes (Kuro-o et al., 1997). In Klotho mice (*kl/kl*), both bone formation and resorption were reduced, indicating a low turnover of bone metabolism resembling human osteoporosis (Kawaguchi et al., 1999). Although neither osteoblasts nor osteoclasts expressed the *kl* gene, ex vivo cultures for osteoblastogenesis and osteoclastogenesis

showed reduced differentiation independently in both lineages. Thus, this model is unique in its correlation with senile osteoporosis in humans, in contrast to the canonical progeroid models.

It is also worth considering the contribution of longevity mouse models to our understanding of the pathophysiology of bone metabolism in normal aging (Migliaccio et al., 1999; Flurkey et al., 2001; Holzenberger et al., 2003). In that regard, IGF is an important factor for bone metabolism, especially bone formation. The critical role of its receptor, IGF1R, in bone modeling has been demonstrated using a conditional knockout strategy (Zhang et al., 2002). Interestingly, heterozygous knockout of IGF1R results in 33% and 16% increases in the mean life-span of female and male animals, respectively (Holzenberger et al., 2003). Together, these results demonstrate that, while a correlation between bone phenotype and life-span is suggested, the issue is complicated and awaits further definition.

3. Bone formation defects mimicking pathogenesis in senile osteoporosis

Low turnover rate or uncoupling between bone resorption and formation in aged bones is often associated with decline in osteoblast function (Bilezikian et al., 2002). Indeed, reduced bone formation is one of the characteristic hallmark features of models for senile osteoporosis. By now, a number of the genes playing critical roles in bone formation have been described in genetically modified mice (Davey et al., 2004). Sca1 is a GPI-anchored membrane protein that is expressed in hematopoietic stem cells, and in a subset of bone marrow stromal cells (Yeh et al., 1986; Stanford et al., 1997). Whereas Sca1 knockout mice have normal bone development, the aged animals (15 months of age) show significant bone loss (Bonyadi et al., 2003). Progenitor and differentiation assays of bone marrow cells in these mice revealed that decreased bone mass is caused by impairment in the self-renewal of mesenchymal progenitors. Stem cell defects in hematopoietic lineages have also been reported in Sca1 knockout mice (Ito et al., 2003). Although multiple aging phenotypes in Sca1 knockout mice have not been reported, this is a good correlative model for senile osteoporosis in humans.

c-Abl, a downstream protein kinase of ATM, functions in DNA repair and oxidative stress response (Kharbanda et al., 1997; Hantschel and Superti-Furga, 2004). Mice deficient for the *Abl* gene also develop osteopenia with reduced bone formation (Li et al., 2000). Ex vivo cultures of osteoclastogenesis were not affected, and the number of osteoclasts was similar to that of wild-type littermates. However, the number of progenitors in bone marrow were decreased, and the differentiation of osteoblasts from *Abl* knockout mice was significantly impaired (Li et al., 2000). Using osteoblast cultures, distinct roles in the oxidative stress response between c-Abl and ATM, have been demonstrated (Li et al., 2004). Although decreased expression of peroxiredoxin 1 due to downregulation of PKCδ was observed upon arsenate-induced oxidative stress in osteoblasts from ATM knockout mice, expression of the redox protein, through the upregulation of PKCδ, was increased in the cells derived from Abl knockouts. The opposing roles of Abl and ATM gene prod-

ucts in the oxidative stress response, as shown in these knockout mice, may cause similar but slightly different bone phenotypes. Nagai et al. demonstrated that oxidative stress plays a crucial role in the aging symptoms of Klotho mice as well (Nagai et al., 2003). Life-span shortening and age-related defects have been reported in mice lacking *MsrA* or *Prdx1*, which encodes methionine sulfoxide reductase or peroxiredoxin 1, respectively (Moskovitz et al., 2001; Neumann et al., 2003). Both genes play important roles in the oxidative stress response, by possessing anti-reactive oxygen species (ROS) activity. Oxidative stress-induced ROS activity often causes damage to DNA, suggesting that genomic stability and the oxidative stress response may share some common pathways in the aging phenotype. While the bone phenotype in these mutant mice has not been described, it is interesting to speculate that loss of these gene products could also play a role in bone pathogenesis.

4. Concluding remarks

Skeletal malformation and severe developmental defects in bone modeling have not been pronounced in the models of premature aging, suggesting that cell differentiation is not seriously arrested. Regeneration from stem cells is an essential step in tissue metabolism and maintenance, including bone remodeling, and the defects in self-renewal of stem cells cause degenerative diseases due to the resultant deviation

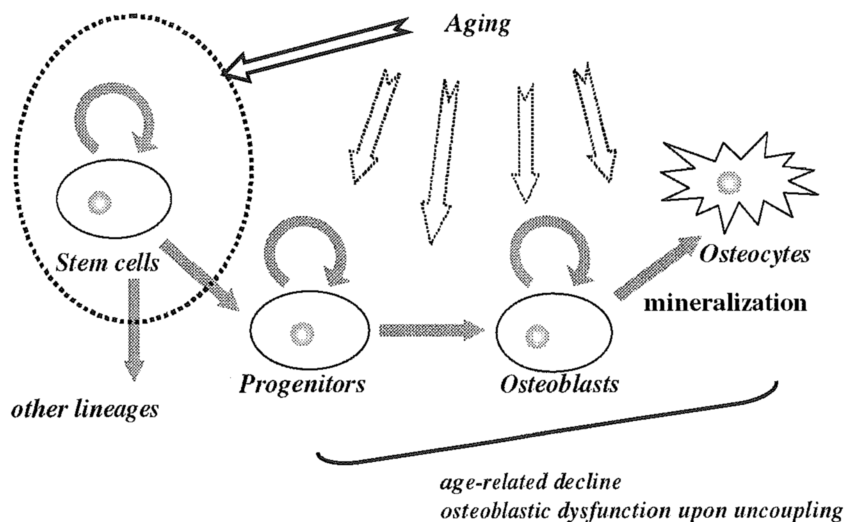

Fig. 2. Schematic presentation of osteogenesis and aging. Observations in naturally aged laboratory animals and ATM knockout mice suggest that the key to understanding aging and premature aging pathogenesis may be self-renewing stem cells. In these models, the pathway involving p53 (Fig. 1) upregulates the genes responsible for cell cycle arrest and/or apoptosis, lowering the regenerative potential necessary for homeostasis and tissue repair. While the mechanisms responsible for aging are largely unknown, the existing models suggest that there are common pathways, which may help in our understanding of the aging phenotype.

of tissue metabolism (Fig. 2). The possibility cannot be ruled out that the aging phenotype is caused by dysfunction in these programs, which become active postnatally or after maturation. As in the case of *Abl* knockout mice, the defect in osteoblastic differentiation, probably due to deregulated response to oxidative stress, also causes symptoms mimicking senile osteoporosis. On the other hand, a number of age-dependent declines in tissue function, such as mineral homeostasis, also affect bone metabolism. Since the characterization of metabolic parameters and tissue functions are often limited in studies in mice, there is a possibility that the apparent age-related decrease in bone mass is secondary to metabolic dysfunctions, rather than from cell autonomous defects. In conclusion, while much information from these mouse models has been gathered over the last decade, studies on senile osteoporosis using these models may be just beginning.

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RumMAGE-D the Members: Structure and Function of a New Adaptor Family of MAGE-D Proteins

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MAGE genes were first described as cancer-testis antigens, which are silenced in normal adult tissues but aberrantly expressed in tumor cells. The short peptides, derived from the degradation of MAGE transcripts, are the source of antigens that cause tumor rejection reactions when presented in the context of major histocompatibility complex. The recent discovery of a subset of genes that contain the structurally conserved MAGE homology domain (MHD) has accelerated the investigation into the normal function of MAGE genes. This new type of MAGE gene is normally expressed in embryonal and adult tissue, especially the brain. MAGE-D1, also known as NRAGE or Dlxin-1, functions as an adaptor protein that mediates multiple signaling pathways, including NGFR (p75NTR) and UNC5H1-induced apoptosis and Dlx/Msx-mediated transcription. Loss of a different MAGE family member, Necdin, which works as a cell cycle regulator, may play a role in the pathogenesis of Prader-Willi syndrome, a neurobehavioral disorder. In this article, the authors discuss recent findings concerning the structure and function of new MAGE genes, primarily focusing on MAGE-D1. Because some MAGE-D subfamily proteins share significant homology within the MHD, these recent discoveries on MAGE-D1 may give insight into the function of other MAGE-D proteins.

Key Words: MAGE-D; NGFR (p75NTR); UNC5H; Apoptosis; Dlx/Msx; Necdin

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INTRODUCTION

What Is MAGE Gene Family?

The initial report of MAGE (melanoma-associated antigen) gene was described in 1991 by Van den Brukken et al., as the identification of a novel gene encoding a tumor rejection antigen presented by human melanoma cells (1). Many structurally similar genes have been cloned thereafter, and they form a large cluster of genes called the MAGE family (reviewed in 2 and 3). In adult tissue, these "classical" MAGE genes, recently termed "type I MAGE" by Barker and Salehi (3), are silenced by DNA methylation except for male germ cells (4). The aberrant transcription of MAGE genes is induced, however, in melanoma and many other tumors (1). Therefore, they have been categorized as so-called "cancer-testis antigens." In MAGE-expressing tumors, short peptides derived from the degradation of MAGE transcripts are processed in antigen-presenting cells and then presented in the context of the major histocompatibility complex (MHC) molecule. Host cytotoxic T-lymphocytes recognize these presented peptides as tumor-specific antigens, resulting in the tumor rejection reaction (reviewed in 5). Although many studies have focused on the nature of this antigenicity of these MAGE peptides and the potential for harnessing this in cancer immunotherapy, it is only recently that studies have described the physiological functions of MAGE family members in normal tissue and in development.

Recent studies reveal growing numbers of new MAGE genes, termed by Barker and Salehi as "type II MAGE." These genes are expressed both during development and in adult tissue (3) and appear to play a wide variety of functions in normal cells (Table 1).

This article reviews the molecular and genetic studies describing type II MAGE genes, focusing primarily on MAGE-D1. In addition, to avoid confusion, the nomenclatures for MAGE-D genes/proteins were unified as indicated in Table 1.

Identification of MAGE-D1

By using a variety of approaches, MAGE-D1 was independently identified by three groups. MAGE-D1 was originally discovered as a novel human MAGE gene in the course of representational difference analysis using cDNA from bone marrow stromal cells (6). Shortly thereafter, insights into the functional role of this new MAGE family member were reported. It was found that the rat homolog of MAGE-D1, named NRAGE, binds the NGFR (Neurotrophin receptor) and regulates neuronal apoptosis (7). It was also discovered that the mouse homolog of MAGE-D1, named Dlxin-1 (Dlx-interacting protein-1), associates with the homeoprotein Dlx5, regulating its transcriptional function (8).

The MAGE-D1 gene encodes 775 amino acids (in mouse), and the amino acid sequence is highly conserved between human, mouse, and rat. MAGE-D1

Table 1: Type II MAGE genes/proteins and their predicted roles in mammalian cells.

Name	Alternative Name(s)	Chromosome	Interacting proteins	Predicted function	Ref.
MAGE-D1	NRAGE, Dlxin-1	Xp11.23	NGFR, UNC5H1, Necdin, Dlx/Msx, IAPs, Ror2, Praja1	Apoptosis, cell cycle, transcription	7,8,32,37,48,49,50
MAGE-D2	JCL-1, BCG1	Xp11.2			2
MAGE-D3	Trophinin, Magphinin	Xp11.22	(tastin)	Implantation, gematogenesis	2,12,14
MAGE-D4	(MAGE-E1)	Xp11			13
MAGE-E1	DAMAGE	Xq13.1	α-dystrobrevin	Signal transduction	15
MAGE-F1		3q13			16
MAGE-G1	Necdin-like 2	15q11-12	E2F1, NGFR	Cell cycle	57
MAGE-H1	APR-1	Xp11.22	NGFR		66
MAGE-L2	Necdin-like 1	15q11-12		Pathogenesis of PWS(?)	64
Necdin		15q11-12	E1A, E2F1, SV40 large T, p53, NEFA, NGFR, Praja1	Cell cycle, calcium homeostasis, pathogenesis of PWS	54,55,56,57,58,59,60, 61,62,66

has similarity to other MAGE family proteins at the C-terminus, termed the MAGE homology domain (MHD). The sequence of MHD is a common feature characteristic of the MAGE family proteins. It can be found in mammalian species and also in databases of *Xenopus*, *Drosophila* (9), and Zebrafish (10). Proteins containing MHD are also present in some vegetal species such as *Arabidopsis thaliana,* although, to date, there is no homologous sequence in *Caenorhabditis elagans* or yeast. A unique characteristic of MAGE-D1 is the 25 hexapeptide repeat with the consensus sequence of WQXPXX present in the middle part of the protein. These repeats are the putative binding domain for Dlx5 and show no homology with other sequences in the database.

As for genomic structure, the MAGE-D1 gene is composed of 13 exons, and the MHD is encoded by multiple exons rather than the single exon, which encodes the MHD in other MAGE genes (2). These findings indicate that the other MAGE genes may have originated by gene duplication from an ancestral MAGE-D1 gene.

The expression of MAGE-D1 is detected in most adult tissues, predominantly in brain, in contrast to the type I MAGE whose normal expression is restricted to male germ cells (2,8). MAGE-D1 is also expressed in the developing embryo in brain, spinal cord, muscle, and cartilaginous tissue, with strongest expression detected in the central nervous system (11). Although many transformed cell lines and tumor tissues also express MAGE-D1, whether MAGE-D1 is presented by MHC like other MAGE family members is not yet known. The nearly ubiquitous expression of MAGE-D1 in adult tissue and its widespread expression during development bring new perspectives for the analysis of the biological roles of this MAGE gene family member.

MAGE-D Subfamily

There are three other genes that contain sequences that are closely related to the sequence of the MAGE-D1 MHD, namely, MAGE-D2 (2), MAGE-D3 (2,12), and MAGE-D4 (13). However, the homology between MAGE-D proteins is restricted to the region encoding MHD. They are all clustered in chromosome Xq11, and their MHD is composed of multiple exons, which seems to be a unique feature of the MAGE-D subfamily.

The expression of MAGE-D2 is also ubiquitous in adult tissue, but higher expression is found in developing embryo (2). The MAGE-D2 protein is unique among MAGE proteins because there are 12-peptide repeats, unrelated to the WQXPXX motif of MAGE-D1, in the middle part of the protein.

The MAGE-D3 gene encodes the MHD in the middle portion and has 69 tandem repeats of decapeptide sequences in the C-terminal site (12). There are five transcripts encoded by MAGE-D3, namely, magphinin-α, magphinin-β, magphinin-β $\Delta 22$, magphinin-γ, and trophinin, which are formed by the alternate use of exons. Trophinin was previously reported as a membrane adhesion

molecule potentially involved in placental implantation into the uterus (14). Trophinin lacks the MHD probably due to translation from a downstream AUG codon. In contrast, magphinins possess the MHD but lack tandem decapeptide repeats, suggesting that they play a different role than trophinin. Both trophinin and magphinins are expressed in brain and in male and female reproductive organs.

MAGE-D4, initially termed as MAGE-E1, was isolated as a gene specifically up-regulated in human glioma (13). MAGE-D4 shows the closest homology with MAGE-D1 in the MHD region, 78.3% identical and 96.1% similar. Three alternatively spliced variants have been found and one of these forms lacks the MHD. The normal expression of MAGE-D4 is found in neuronal tissue and ovary. The mouse genome does not contain MAGE-D4 orthlogues.

A commonality shared by MAGE subfamilies is the frequent presence of tandem repeats so that despite the fact that the overall amino acid sequence between family members is often poorly conserved, they share this structural feature. Each MAGE-D protein has a distinct tandem repeat as described above. In addition, tandem repeats are seen in other subfamilies of type II MAGE genes. The recently identified DAMAGE/MAGE-E1, which binds to α-Dystrobrevin in the Dystrophin complex, has 30 tandem repeats with the 12-amino acid consensus of A (S/T)EGPSTSVPPT (15). Repeat regions in these type II MAGE proteins may function as domains for protein-protein interactions; for example, MAGE-D1 binds to Dlx5, and trophinin binds to itself via these repeat regions (8,14). In addition, the DAMAGE/MAGE-E1 repeat sequence contains consensus sequences recognized by protein kinases. Thus one possibility is that phosphorylation at these sites regulates interactions by DAMAGE/MAGE-E1 (15). Furthermore, this repeat contains potential PEST sequences that may play a role in targeting the protein for degradation. We note here that there has been confusion in the literature because several genes have been called MAGE-E1 (13), but in the unified nomenclature, DAMAGE is considered the actual MAGE-E1 (2). MAGE-F1, is another member of the type II subfamily of MAGE genes. It contains seven GAG repeats, which encode a stretch of polyglutamic acid (E) residues (16). These repeats are reminiscent of the polyglutamine (Q) repeats that are expanded in neurodegenerative diseases such as Huntington's disease. The significance of the these repetitive sequences within MAGE genes is unknown. Because it has been proposed that the repetitive sequences may be involved in arrest of gene duplication (17,18), one might speculate that expansion of type II MAGE genes may be attenuated by these repeat sequences.

Association of MAGE-D1 with Cell Surface Receptors

Several surface receptors have been reported to associate with MAGE-D1. One of them is NGFR (p75NTR), which belongs to TNF receptor superfamily and plays a crucial role in the developing nervous system and during synaptic

plasticity. Recent studies show that NGFR exerts apparently opposite effects, cell death vs. cell survival, due to the existence of various classes of ligands, a number of partner receptors, and different cytoplasmic interactors (reviewed in 19 and 20).

The canonical class of ligands for NGFR is the neurotrophins (NTs); nerve growth factor (NGF), brain derived neurotrophic factor (BDNF), NT3, and NT4/5. NGFR forms high-affinity receptor for NTs in association with three tyrosine kinase domain receptors, TrkA for NGF, TrkB for BDNF, TrkC for NT3 and NT4/5. When bound to the Trk/p75NTR receptor complex, NTs promote neuronal survival signals via the MAPK, PI3K, and PLC pathways activated by Trk receptors and their substrates (reviewd in 20) (Fig. 1A). Recently, a proform of NGF was shown to be a second class of ligands for p75NTR (21). NTs are synthesized as precursors that can be cleaved proteolytically to produce mature NTs. In some tissues, a substantial proportion of ProNT is not cleaved, suggesting that they could be present physiologically to act as ligands for NGFR. Indeed, proNGF binds p75NTR with high affinity and mediates an apoptotic

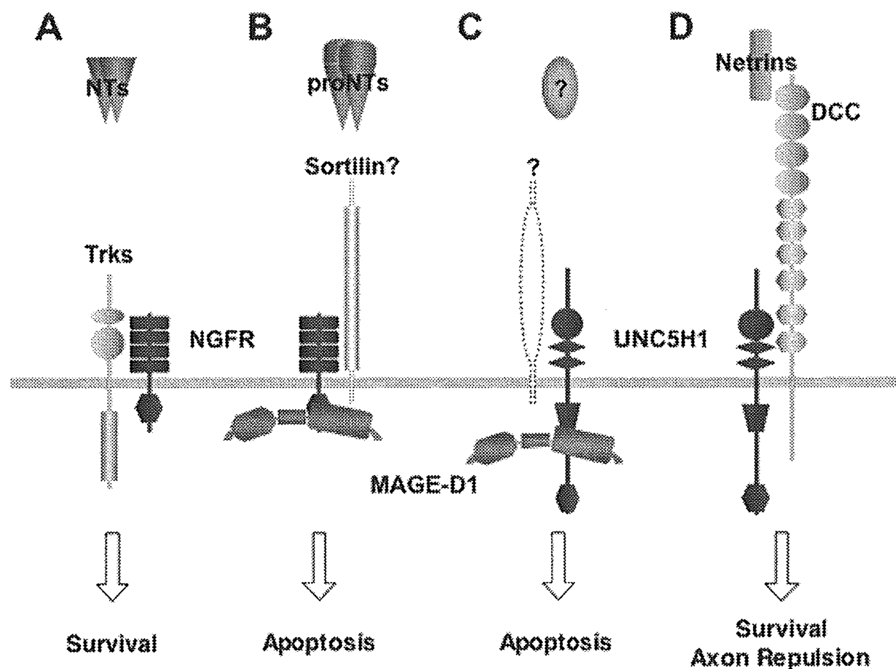

Figure 1: MAGE-D1 associates to NGFR and UNC5H1 and mediates apoptotic signal. (A) Neurotrophins (NTs) elicit neuronal survival signal when they bind to Trk/NGFR receptor complex. (B) Pro-NTs promote apoptotic signal when they bind to NGFR. MAGE-D1 facilitates NGFR-dependent apoptosis by binding to juxtamembrane domain of NGFR, probably in this pro-NTs signaling. (C) MAGE-D1 also exerts UNC5H1-dependent apoptosis by binding to the cytoplasmic region of UNC5H1 including PEST and ZU-5 domain. (D) In the presence of the ligand Netrins and binding partner receptor DCC, UNC5H1 mediates survival signal and axon repulsion.

pathway in neurons and oligodendrocytes (21,22). Although we have no information about the involvement of MAGE-D1 in proNT-dependent apoptosis, it is strongly suggested that the apoptotic signal mediated by p75NTR/MAGE-D1 interaction may be caused by proNTs (Fig. 1B). Commercial, recombinant NTs may be contaminated with proNTs, and this may explain the NT-dependent apoptosis observed in some studies, including the study of MAGE-D1 (7,23).

NGFR is a member of a TNF receptor superfamily characterized by cysteine-rich domains extracellularly and an intracellular C-terminal death domain. In the absence of Trk receptors, NGFR mediates an apoptotic signal. Although the signaling cascades activated by NGFR to mediate apoptosis remain poorly understand, studies suggest that it involves multiple cytoplasmic interactors that act as adaptors for these apoptotic pathways (reviewd in 20). MAGE-D1 is one of these adaptor proteins, and it binds to the juxtamembrane region, not the death domain of NGFR. Co-expression of MAGE-D1 with NGFR facilitates NGF and NGFR-dependent apoptosis in MAH symphathoadrenal cell line that lacks TrkA. Overexpression of MAGE-D1 disrupts the NGFR/TrkA complex, indicating that the interaction between NGFR and MAGE-D1 or TrkA is mutually exclusive (7). MAGE-D1 activates the JNK and caspase pathway and subsequently leads to apoptotic cell death via a mitochondrial apoptotic pathway (24). On internalization after ligand binding, MAGE-D1 co-localizes with NGFR, but not with TrkA, in early endosome, supporting the idea that neurotrophin/NGFR/MAGE-D1 complex may transmit signals intracellularly as part of signaling endosomes (25). Taken together, the results indicate that MAGE-D1 may be an intracellular mediator of NGFR-dependent apoptosis.

In addition to the well-established role of enhancing the ability of Trk receptors to bind and respond to NTs, the interaction of NGFR with additional transmembrane proteins was recently demonstrated. Sortilin is one of these coreceptors. It is a member of the VPS10 family and plays a role in intracellular trafficking (26) Sortilin also binds NGFR and proNGF, forming a complex at the cell surface that is responsible for the pro-apoptotic effects of proNGF (27). In addition, NGFR forms a receptor complex with the Nogo receptor (NgR), a GPI-anchored protein, and LINGO-1, a brain-specific transmembrane protein. Together, they participate in mediating the effects of myelin-based growth inhibitors such as myelin-associated protein (MAG) (28,29). Binding of myelin-associated proteins to NgR in NgR/LINGO-1/NGFR receptor complex activates RhoA, which is required as an inhibitory signal for neurite outgrowth (30). The role of MAGE-D1, however, in neurite outgrowth through NGFR signaling remains to be determined.

Analysis of the temporal and spatial expression of MAGE-D1 and NGFR during murine embryogenesis (31) revealed that MAGE-D1 protein is expressed throughout the neural tube during early neuronal development. This expression becomes restricted at later stages of neurogenesis, to the ventricular zone, subplate, and cortical plate. Significantly, the expression of MAGE-D1 is not

restricted to regions of NGFR expression, suggesting that MAGE-D1 plays additional roles during neuronal development, perhaps in association with other signaling receptors.

One such candidate signaling receptor is UNC5H1, a netrin-1 receptor belonging to the immunoglobulin superfamily receptors. MAGE-D1 and UNC5H1 display overlapping expression in embryonal neural tissue, including outer layers of the cortex, cortical plate, marginal zone, hippocampus, olfactory bulb, and motor neurons. The juxtamembrane sequence of UNC5H1, containing a PEST and ZU-5 sequence, binds MAGE-D1 and through this interaction induces apoptosis (Fig. 1C) (32). Like NGFR, UNC5 receptors also have a co-receptor, DCC. Together DCC and UNC5 mediate netrin-1-dependent repulsion of neurons and their axons, but the signaling events underlying this function (e.g., whether MAGE-D1 is involved in this cell migration) are not known (33). It has also been reported that UNC5Hs and DCC mediate apoptosis in the absence and not the presence of ligand Netrin-1 (34,35). For UNC5Hs, this death requires the C-terminal death domain and appears to be distinct from the apoptosis induced by the interaction of MAGE-D1 with UNC5H1 (34).

Here we note the striking structural and functional similarity between NGFR and UNC5H1 (Fig. 1). They both bind to MAGE-D1 through juxtamembrane sequences that contain a PEST sequence. They both have C-terminal death domains that play a role in inducing apoptotic pathways that are distinct from the apoptotic pathways induced by their association with MAGE-D1. They both undergo cleavage and release their cytoplasmic domains into the cytoplasm (32,35,36). The observation that MAGE-D1 also functions as a transcriptional regulator (8) makes it tempting to speculate that the intracellular domains of NGFR and UNC5H1 use their interaction with MAGE-D1 to signal to the nucleus.

A receptor tyrosine kinase Ror2 also associates with MAGE-D1 (37). The involvement of Ror2 in Dlx/Msx signaling mediated by MAGE-D1 is discussed in the next paragraph.

Interaction to Homeodomain Proteins

MAGE-D1 was also identified as Dlxin-1 (Dlx-interacting protein-1) because it interacts with Dlx5, a member of the homeodomain transcription factor family (8). Dlx5 is normally expressed in developing brain and skeletal elements. Its expression is induced by bone morphogenetic proteins (BMPs), and it is involved in bone formation by regulating essential genes for osteoblastic differentiation such as osteocalcin and Runx2 (38,39). Mice bearing a targeted disruption of the Dlx5 gene display severe craniofacial abnormalities, suggesting that Dlx5 plays a pivotal role in neuronal and mesenchymal cell differentiation, particularly in the craniofacial area (40,41). Although MAGE-D1, itself, does not bind DNA, transfection and reporter gene assays reveal that it enhances Dlx5-dependent transcriptional activity (Fig. 2A) (8).

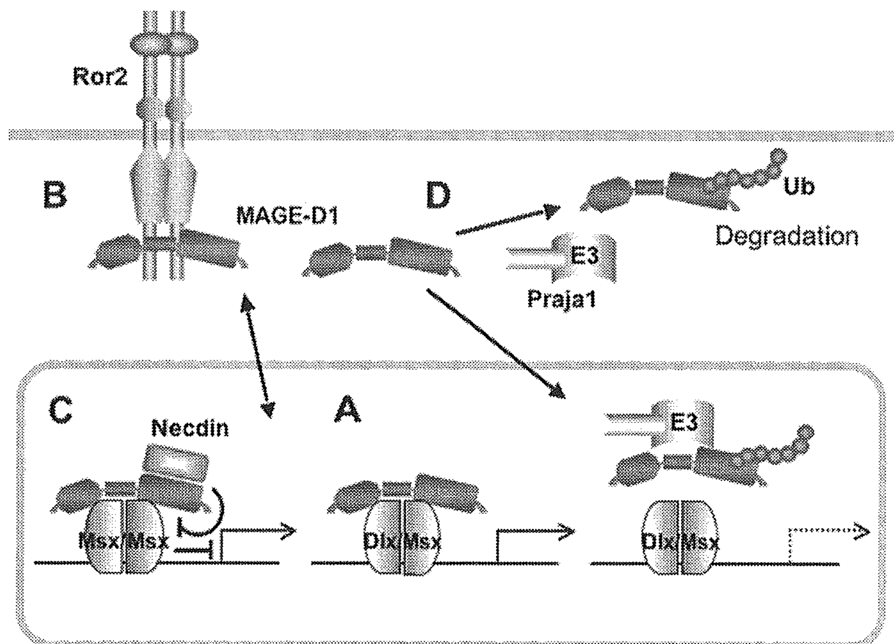

Figure 2: MAGE-D1 and Dlx/Msx signaling. (A) MAGE-D1 binds to Dlx/Msx homeodomain transcriptional factors and regulates Dlx/Msx-dependent transcription in additive manner. (B) Ror2 associates and recruits MAGE-D1 at membranous compartment, thereby inhibits nuclear co-localization of MAGE-D1 and Msx2 and reduces their transcriptional activity. (C) Co-expression of Necdin and MAGE-D1 abolishes Msx2-dependent suppression of transcription. MAGE-D1 acts as an adaptor to link Necdin to Msx2. (D) Praja1 promotes ubiquitination and proteasome-dependent degradation of MAGE-D1. Ubiquitinated MAGE-D1 reduces Dlx/Msx-dependent transcription possibly by conformational change and detachment of the transcriptional complex from DNA, or by recruiting the nuclear co-repressors within the complex.

MAGE-D1 also binds to other Dlx/Msx family proteins including Msx2. During late embryogenesis, strong expression of MAGE-D1 mRNA is detected overlapping with Msx2 expression in interdigital soft tissue (8,42). Although the precise mechanism of their action is currently unknown, BMPs, BMP receptor, and BMP-inducible Msx2 are all co-expressed interdigitally and suspected to play a crucial role in interdigital cell death in embryonic limb (42,43). Taken together, MAGE-D1 may contribute to interdigital apoptosis in collaboration with Msx2.

Modulation of Dlx/Msx signaling by MAGE-D1 is affected by both the expression level of MAGE-D1 protein and its intracellular localization. For example, the cytoplasmic domain of Ror2 associates with and recruits MAGE-D1 to an intracellular membranous compartment, thereby inhibiting the nuclear co-localization of Dlxin-1 with Msx2, which in turn regulates Msx2-dependent transcriptional activity (Fig. 2B) (37). Ror2 plays a crucial role in developmental morphogenesis, particularly in skeletal and cardiac development (44). In

E10.5 and E12.5 mouse embryo, Ror2 and MAGE-D1 exhibit similar expression patterns especially in limb buds and the pharyngeal arch. It is tempting to speculate that Ror2 may function in conjunction with BMP signaling to modulate interdigital apoptosis by regulating nuclear co-localization of Dlxin-1 and Msx2. It is of interest that mutations in human Ror2 gene cause some skeletal abnormalities such as brachydactyly (45). Recently, the ligands of Xenopus Ror2 (Xror2) were identified as Xenopus Wnt proteins (Xwnts). During early Xenopus embryogenesis Xror2 participates in mesoderm/neuroectoderm movement in cooperation with Xwnts (46). Although the role of MAGE-D1 in non-canonical Wnt signaling via Ror2 receptor remains to be determined, there may be novel cross-talk between BMPs, (pro)NTs, and Wnt signaling pathways intersecting at MAGE-D1.

Recently, it was reported that Necdin is also physically and functionally associated with Msx2 (47,48). Expression profiling of two clones derived from a mesoangioblast cell line, one displaying more and one less differentiated characteristics, revealed that Msx2 and Necdin were expressed in the more differentiated clone at a level 100 times greater than their expression in less-differentiated, more stem-like cell (47). In analyzing this result, it was found that both genes are up-regulated on TGF-β-induced differentiation of mesoangioblast cells to smooth muscle cells. Furthermore, Msx2 and Necdin were found to be both necessary and sufficient in activating the expression of a number of smooth muscle specific genes. In other studies on myoblast differentiation, it was shown that Msx2-dependent suppression of C2C12 myoblast differentiation was abolished by the co-expression of Necdin and MAGE-D1. MAGE-D1 acts as an adaptor to link Necdin to Msx2, suggesting that these MAGE proteins cooperatively play a role in cell differentiation through the regulation of Msx function (48) (Fig. 2C).

Praja1, a RING-finger protein, is another MAGE-D1-interacting protein (49). Praja1 has an E3 (ubiquitin ligase) activity and promotes ubiquitin-proteasome-dependent degradation of MAGE-D1 (Fig. 2D). Proteomic analysis of the MAGE-D1 protein complex reveals that either Praja1 or its related protein, Praja2/Neurodap1, is part of the complex (AS and KW, unpublished data). Overexpression of Praja1 also reduces the levels of Msx2, which can be found in a complex with both MAGE-D1 and Praja1. This degradation may play a regulatory role in Dlx/Msx transcription because wild type, but not a RING-finger mutant of Praja1, inhibits Dlx5-dependent transcriptional activity. Of interest is the observation that ubiquitination of MAGE-D1 per se reduces Dlx5-dependent transcriptional activity without involving proteasome-dependent degradation of MAGE-D1. These experimental results indicate that ubiquitination of MAGE-D1 may regulate the expression level and function of both MAGE-D1 and MAGE-D1-interacting proteins.

Apoptosis- or Cell Cycle Regulating Proteins Associated with MAGE-D1 or Necdin

ITA and XIAP, which both belong to the family of inhibitor of apoptosis proteins (IAPs) also are reported to associate with MAGE-D1 (50). IAPs promote anti-apoptotic function through inhibiting both initiator and effector caspases (reviewed in 51). The interaction of IAPs and NRAGE is exerted between the RING domain of IAPs and N-terminal of the MAGE-D1, respectively. MAGE-D1 augments IL-3 withdrawal-dependent apoptosis in 32D cells through binding to XIAP and subsequently inducing the cleavage of XIAP. Recent reports showed that cell cycle arrest induced by overexpression of MAGE-D1, is mediated by p53 through the up-regulation of phosphorylation at Ser392 in the tumor suppressor, although no information concerning the physical interaction between MAGE-D1 and p53 was provided (52). Traube/Che-1/AATF, an Rb-binding protein that activates E2F1 by inhibiting binding of HDAC to Rb (53), also has been identified as a binding protein of MAGE-D1 (AS and KW, unpublished data). Thus, it is suggested that MAGE-D1 plays a role in regulation of cell cycle and/or apoptosis via the interaction to their effector molecules.

Functional binding partners to Necdin have been reported as well. In SaOS-2 cells that lack Rb protein, Necdin associates to E1A, E2F1, and SV40 large T antigen and inhibits cellular proliferation as an Rb-like growth suppressor (54). Necdin also binds to p53 and suppresses p53-dependent transcription (55). In addition, the Ca-binding protein NEFA, which is involved in intracellular Ca2+ homeostasis in neurons, is also reported to be a binding partner of Necdin (56). These results suggest that Necdin may be a cell cycle regulator in proliferating cells and influencing genomic stability and intracellular homeostasis in postmitotic neurons. MAGE-D1 and Necdin play similar roles, but by different mechanisms, in the regulation of cell cycle and apoptosis. Furthermore, roles in cell cycle regulation have been proposed for other type II MAGE proteins (57). Thus there may be tremendous functional redundancy and compensatory actions among those molecules that have not yet been elucidated.

Necdin and Prader-Willi Syndrome

Although there is some evidence that MAGE genes play a role in human disorders, their function in disease settings has not been extensively characterized. Among type II MAGE proteins, there is some evidence that loss of Necdin function, which has been reported to be highly expressed in postmitotic neurons (58), is associated with human neurological disease.

The Necdin gene, NDN, maps to human chromosome 15q11-q13, a region syntenic with mouse chromosome 7 where some maternally imprinted genes are mapped (NDN, SNRPN, IPW, ZNF127, and MAGEL2/NDNL1) (59). Because maternal allele coding of these genes is silenced, paternal deficiency or