Table 1. List of Genes that Had Up-Regulated (Positive) and Down-Regulated (Negative) Expression Related to EMT (PC3 Axis)

Positive			Negative		
Gene Symbol	**Gene Title**	**Representative Public ID**	**Gene Symbol**	**Gene Title**	**Representative Public ID**
Ccng2	cyclin G2	U95826	Bach1	BTB and CNC homology 1	NM_007520
Ccni	cyclin I	NM_017367	Cdc42bpa	Cdc42 binding protein kinase alpha	BM117074
Ctgf	connective tissue growth factor	NM_010217	Dnm1	dynamin 1	L31397
Dock7	Dedicator of cytokinesis 7	BB463580	Foxb1	forkhead box B1	U90538
Dok1	docking protein 1	BC006868	Gprc5c	G protein-coupled receptor, family C, group 5, member C	BC008228
Fgfr1	Fibroblast growth factor receptor 1	M33760	Il13ra1	interleukin 13 receptor, alpha 1	S80963
Gja1	gap junction membrane channel protein alpha 1	BB039269	Kcnk5	potassium channel, subfamily K, member 5	AF319542
Gtpbp4	GTP binding protein 4	AI987834	Kif13a	kinesin family member 13A	AB037923
Hgfac	hepatocyte growth factor activator	NM_019447	Kif17	kinesin family member 17	AW492270
Hoxa3	homeo box A3	BB496114	Mark2	MAP/microtubule affinity-regulating kinase 2	BI686265
Hoxb8 /b7	homeo box B8 / homeo box B7	X13721	Mef2d	myocyte enhancer factor 2D	NM_133665
Il15ra	interleukin 15 receptor, alpha chain	NM_008358	Mrpl51	mitochondrial ribosomal protein L51	AI594880
Irx2	Iroquois related homeobox 2	AF295369	Mxd4	Max dimerization protein 4	BE291523
Itga5	integrin alpha 5	BB493533	Neu1	neuraminidase 1	AI649303
Itgb1	integrin beta 1	BM120341	Rel	reticuloendotheliosis oncogene	NM_009044
Mapkbp 1	Mitogen activated protein kinase binding protein 1	BQ174980	Rgnef	Rho-guanine nucleotide exchange factor	BG069493
Mdm2	transformed mouse 3T3 cell double minute 2	X58876	Rps6kb2	ribosomal protein S6 kinase, polypeptide 2	NM_021485
Ncam1	neural cell adhesion molecule 1	NM_010875	Slc24a1	solute carrier family 24, member 1	BC016094
Pdgfa	platelet derived growth factor, alpha	BB371842	Slc25a19	solute carrier family 25, member 19	AV338420
Prkcc	protein kinase C, gamma	NM_011102	Slc25a22	solute carrier family 25 , member 22	AK018760
Rab23	RAB23, member RAS oncogene family	NM_008999	Slc40a1	solute carrier family 40 , member 1	AF226613
Rasa3	RAS p21 protein activator 3	NM_009025	Stat1	signal transducer and activator of transcription 1	AW214029
Rb1	retinoblastoma 1	NM_009029	Tgfa	transforming growth factor alpha	M92420
Sbno1	Sno, strawberry notch homolog 1	BC023136	Ubp1	upstream binding protein 1	NM_013699
Slc1a4	solute carrier family 1, member 4	BB277461	Usp12	ubiquitin specific protease 12	AF441835
Slc34a1	solute carrier family 34 , member 1	AI788646			
Slc4a7	Solute carrier family 4, member 7	AW555750			
Slc7a2	solute carrier family 7, member 2	M62838			
Ube1y1	ubiquitin-activating enzyme E1, Chr Y 1	X62581			
Vegfa	vascular endothelial growth factor A	NM_009505			
Wnt6	wingless-related MMTV integration site 6	NM_009526			
Wnt7b	wingless-related MMTV integration site 7B	W29605			

drug delivery for specific anti-cancer drugs, especially those affecting metastasis. Progress in understanding EMT has been an exercise in coming to appreciate the level of complexity required for changing cellular identity. The mecha-

nism of EMT highlights an integration of nuclear regulation and network signaling with alterations in the microenvironment to create a moving cell; in this sense, basic concepts based on EMT mechanisms would thus hold great promise for regenerative medicine.

GET OP9 CELLS DEAD TO RIGHTS

The concept of regenerative medicine refers to the cell-mediated restoration of damaged or diseased tissue. Candidate cell sources for tissue regeneration include ES cells, fetal cells, and adult cells such as marrow stromal cells, each of which has both advantages and drawbacks. Clinical trials with marrow stromal cells have been performed in patients with osteogenesis imperfecta and osteoporosis, and marrow stromal cells are expected to be a good source of cell therapy.

Bone marrow-derived stem cells can be transdifferentiated into multilineage cells, such as muscle [30] from mesoderm, lung [31] and liver [31, 32] from endoderm, and brain [33-36] and skin [31] from ectoderm. Somatic stem cells are more desirable than ES cells for cell therapeutics because of ethical considerations and the possible immunologic rejection of ES cells. Mesenchymal stem cells have become the most popular somatic stem cells in medicine and biology, not least because of their high reproductive capability *in vitro*.

Chondrocytes differentiate from mesenchymal cells during embryonic development [37], and the phenotype of the differentiated chondrocyte is characterized by the synthesis, deposition, and maintenance of cartilage-specific extracellular matrix molecules, including type II collagen and aggrecan [38-40]. The phenotype of differentiated chondrocytes is rapidly lost since it is unstable in culture [41-44]. This process is referred to as 'dedifferentiation' and is a major impediment to the use of mass cell populations for therapy or tissue engineering of damaged cartilage. When isolated chondrocytes are cultured in a monolayer at low density, the typical round chondrocytes morphologically transform into flattened fibroblast-like cells, with profound changes in biochemical and genetic characteristics, including reduced synthesis of type II collagen and cartilage proteins [45].

We established several stromal cells from murine bone marrow cultures [46]. One of them, KUSA-A1 cells, displays osteogenetic characteristics *in vitro* and *in vivo*. In order to clarify the specific gene expression profile of KUSA-A1, other established stromal cells, KUM5, 9-15c, KUSA-O, H-1/A [47], and mouse embryonic fibroblasts, we compared the expression levels of approximately 23,000 genes by using the Affymetrix gene chip oligonucleotide arrays. Of the 23,000 genes represented on the gene chip, chondrocyte-specific or -associated genes such as type II collagen α1, type XI collagen α1, Sox9, proline arginine-rich end leucine-rich repeat, and cartilage oligomeric matrix protein are more strongly expressed in KUM5 cells than in other marrow-derived mesenchymal cells. Does a gene expression pattern reflect the character of the cells *in vitro* and/or *in vivo*? – The answer is yes: KUM5 cells generate hyaline cartilage and exhibit endochondral ossification *in vitro* (Fig. (3B)) and *in vivo* [48].

Surprisingly, OP9 cells [49] also express these chondrocyte-specific or -associated genes at higher levels: the type II collagen α1, and type XI collagen α1 genes are expressed in OP9 cells at more than 10-fold higher levels than in 9-15c, KUSA-O, H-1/A, primary embryonic fibroblasts, or even KUM5 chondroblasts. In addition, expression of 'structural proteins' on Gene Ontology, including the extracellular matrix, is much higher by OP9 and KUM5 cells than by non-chondrogenic cells such as KUSA-A1, H-1/A, and 9-15c, implying that the OP9 and KUM5 cells are mainly engaged in synthesizing extracellular matrix. We also performed hierarchical clustering and PCA, based on the microarray data (Fig. (3A)). KUM5 and OP9 cells are grouped into the same subcategory and can clearly be separated from other stromal cells based on the expression data of cell surface markers and cell-type-specific genes, implying that KUM5 and OP9 cells have chondrogenic potential.

Are OP9 cells chondroblasts *in vitro* and/or *in vivo*? - the answer, again, is yes; OP9 cells are induced into the chondrogenic lineage by the pellet culture method (Fig. (3C)), and the OP9 pellets (micromasses) implanted in mice form the type II collagen-positive hyaline cartilage [48]. OP9 cells are derived from macrophage colony-stimulating factor-deficient osteopetrotic mice, and have also been used as feeder cells for embryonic stem cells [50-52]. The cells identified as a key participant in regulating the number of adult stem cells or hematopoietic stem cells are now considered to be of an osteoblastic lineage [53, 54]. OP9 cells have been recognized as a niche-constituting preadipocyte; however their true face is a chondroblast. We have two different types of cells, osteoblasts (KUSA-A1) and chondroblasts (OP9 and KUM5), showing distinctive *in vivo* characteristics. The unique characteristics of these cells provide an opportunity to analyze the process of membranous ossification and endochondral ossification. These cells are useful candidate cell sources, in addition to dedifferentiated chondrocytes obtained from cartilage for transplantation in osteoarthritis and rheumatoid arthritis.

GENE EXPRESSION PROFILING AND MEDICAL SCIENCE

Recently, gene expression profiling has been successfully used to predict outcomes in some types of malignant diseases [55-61] and, additionally, to assess drug discovery screening [62]. In reproductive and regenerative medicine, it is important to identify biomarkers that will establish the isolation, selection and expansion of stem cells *in vitro* to allow their use for cell therapy. On the road map for translational medicine-- often referred to as bench to bedside research--, stem cell therapy is a prime destination. Stem cells have not taken on the identity of any specific cell type and are not yet committed to any dedicated function; they can divide extensively or indefinitely, and may be induced to give rise to one or more specialized cell types. Stem cells derived from bone marrow can replace heart muscle lost as a result of a heart attack, and can improve cardiac function. Injecting bone-marrow stem cells into an injured heart potentially represented new therapy, triggering the launch of numerous clinical studies to investigate the effect of directly injecting these cells into the damaged heart muscle of patients following a

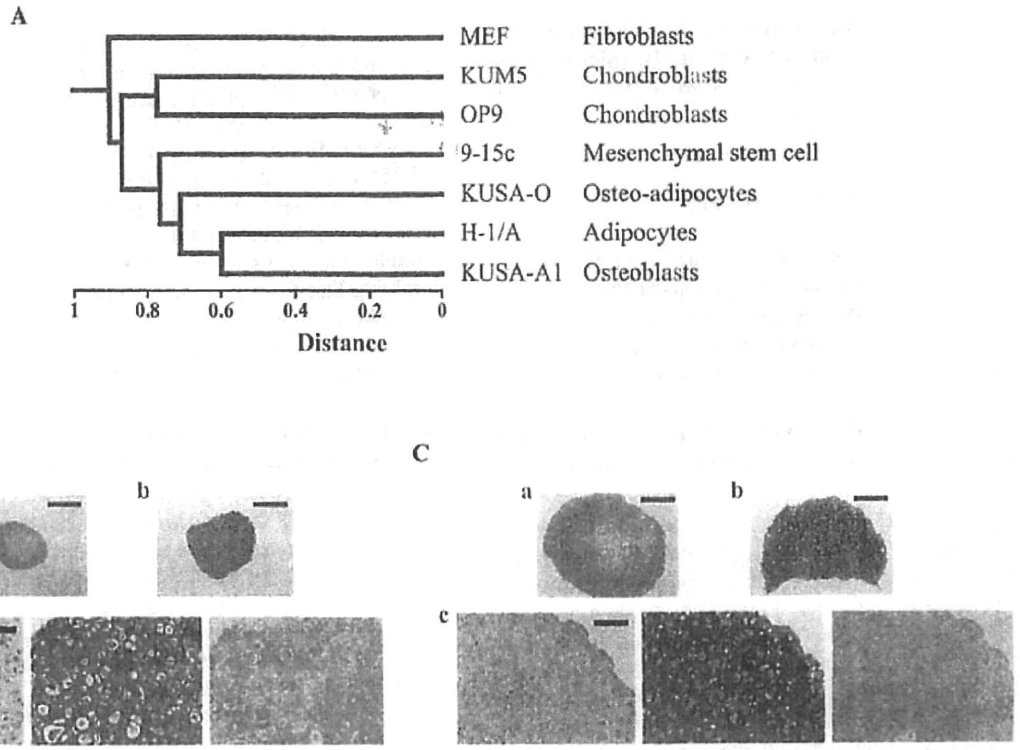


Fig. (3). Expression profiling and *in vitro* chondrogenesis of KUM5 and OP9 cells.

A. Dendrogram revealing clustering profile of six marrow stromal cells and mouse embryonic fibroblast (MEF) using 244 surface marker genes.

B. a, b: Toluidine blue stained section of KUM5 chondrogenic nodules in the pellet culture exposed TGF-β3 and BMP-2 for 1 (a) or 3 (b) weeks. Scale bars: 500 μm. c: Higher magnification of KUM5 chondrogenic pellet exposed to TGF-β3 and BMP-2 for 3 weeks. Left panel: hematoxylin and eosin stain; center panel: toluidine blue stain; right panel: alcian blue stain. Scale bars: 100 μm.

C. a, b: Toluidine blue stained section of OP9 chondrogenic nodules in the pellet culture exposed TGF-β3 and BMP-2 for 1 (a) or 3 (b) weeks. Scale bars: 500 μm. c: Higher magnification of OP9 chondrogenic pellet exposed to TGF-β3 and BMP-2 for 3 weeks. Left panel: hematoxylin and eosin stain; center panel: toluidine blue stain; right panel: alcian blue stain. Scale bars: 100 μm.

(Modified from Sugiki *et al.* J Cell Biochem, 2007, 100, 1240 [48]).

heart attack [63]. The scientific underpinnings of the ongoing human studies have been established. Now is the time to search for the presence of naturally occurring, authentic pluripotent cells and to identify and dissect the signals that guide their migration, self-renewal and differentiation. Furthermore, we need to commit the necessary time and resources to identify the best stem cells for cell therapy to translate.

CONCLUSION

Here, the expression pattern has been correlated with molecular structure descriptor; this consistency indicates that the expression profiling is valid. Consequently, understanding the global gene network that governs the pluripotency and self-renewal of stem cells is an important first step towards the experimental manipulation of cellular developmental potency. The cell potency is a fundamental concept in developmental biology and stem cell biology, providing a conceptual framework of sequential transition from totipotent fertilized eggs to pluripotent embryonic stem cells and stem cells to terminal differentiated cells. The global expression profiling can help to delineate the global architecture and dynamics of a gene regulatory network such as Oct4-regulated gene networks in mouse ES cells [64].

ACKNOWLEDGEMENTS

The authors wish to thank Yoriko Takahashi for helpful discussions, and Kayoko Saito for secretarial assistance.

This study was supported by grants from the Ministry of Education, Culture, Sports, Science, and Technology (MEXT) of Japan; the Ministry of Health, Labour and Welfare Sciences Research Grants; by a Research Grant on Health Science focusing on Drug Innovation from the Japan Health Science Foundation; by the program for promotion of Fundamental Studies in Health Science of the Pharmaceuticals and Medical Devices Agency; by a Research Grant for Cardiovascular Disease from the ministry of Health, Labour and Welfare; and by a Grant for Child Health and Development from the Ministry of Health, Labour and Welfare.

FOOTNOTE

Fig. (**1**) is prepared from ref. [18] with permission from Elsevier.

Fig. (3) is prepared from ref. [48] with permission from Wiley-Liss, Inc.

REFERENCES

[1] Bryder, D.; Rossi, D.J.; Weissman, I.L. Hematopoietic stem cells: the paradigmatic tissue-specific stem cell. *Am. J. Pathol.*, 2006, *169*, 338-346.

[2] Alison, M.; Sarraf, C. Hepatic stem cells. *J. Hepatol.*, 1998, *29*, 676-682.

[3] Watt, F.M. Epidermal stem cells: markers, patterning and the control of stem cell fate. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, 1998, *353*, 831-837.

[4] Potten, C.S. Stem cells in gastrointestinal epithelium: numbers, characteristics and death. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.*, 1998, *353*, 821-830.

[5] Gage, F.H. Mammalian neural stem cells. *Science*, 2000, *287*, 1433-1438.

[6] Jiang, Y.; Vaessen, B.; Lenvik, T.; Blackstad, M.; Reyes, M.; Verfaillie, C.M. Multipotent progenitor cells can be isolated from postnatal murine bone marrow, muscle, and brain. *Exp. Hematol.*, 2002, *30*, 896-904.

[7] Jiang, Y.; Jahagirdar, B.N.; Reinhardt, R.L.; Schwartz, R.E.; Keene, C.D.; Ortiz-Gonzalez, X.R.; Reyes, M.; Lenvik, T.; Lund, T.; Blackstad, M.; Du, J.; Aldrich, S.; Lisberg, A.; Low, W.C.; Largaespada, D.A.; Verfaillie, C.M. Pluripotency of mesenchymal stem cells derived from adult marrow. *Nature*, 2002, *418*, 41-49.

[8] Fridenshtein, A. [Stromal bone marrow cells and the hematopoietic microenvironment]. *Arkh. Patol.*, 1982, *44*, 3-11.

[9] Ko, M.S. Embryogenomics: developmental biology meets genomics. *Trends Biotechnol.*, 2001, *19*, 511-518.

[10] Ivanova, N.B.; Dimos, J.T.; Schaniel, C.; Hackney, J.A.; Moore, K.A.; Lemischka, I.R. A stem cell molecular signature. *Science*, 2002, *298*, 601-604.

[11] Ramalho-Santos, M.; Yoon, S.; Matsuzaki, Y.; Mulligan, R.C.; Melton, D.A. "Stemness": transcriptional profiling of embryonic and adult stem cells. *Science*, 2002, *298*, 597-600.

[12] Schena, M.; Shalon, D.; Davis, R.W.; Brown, P.O. Quantitative monitoring of gene expression patterns with a complementary DNA microarray. *Science*, 1995, *270*, 467-470.

[13] Tanaka, T.S.; Kunath, T.; Kimber, W.L.; Jaradat, S.A.; Stagg, C.A.; Usuda, M.; Yokota, T.; Niwa, H.; Rossant, J.; Ko, M.S. Gene expression profiling of embryo-derived stem cells reveals candidate genes associated with pluripotency and lineage specificity. *Genome Res.*, 2002, *12*, 1921-1928.

[14] Brown, P.O.; Botstein, D. Exploring the new world of the genome with DNA microarrays. *Nat. Genet.*, 1999, *21*, 33-37.

[15] DeRisi, J.L.; Iyer, V.R.; Brown, P.O. Exploring the metabolic and genetic control of gene expression on a genomic scale. *Science*, 1997, *278*, 680-686.

[16] Iyer, V.R.; Eisen, M.B.; Ross, D.T.; Schuler, G.; Moore, T.; Lee, J.C.; Trent, J.M.; Staudt, L.M.; Hudson, J., Jr.; Boguski, M.S.; Lashkari, D.; Shalon, D.; Botstein, D.; Brown, P.O. The transcriptional program in the response of human fibroblasts to serum. *Science*, 1999, *283*, 83-87.

[17] Carter, M.G.; Hamatani, T.; Sharov, A.A.; Carmack, C.E.; Qian, Y.; Aiba, K.; Ko, N.T.; Dudekula, D.B.; Brzoska, P.M.; Hwang, S.S.; Ko, M.S. In situ-synthesized novel microarray optimized for mouse stem cell and early developmental expression profiling. *Genome Res.*, 2003, *13*, 1011-1021.

[18] Hamatani, T.; Carter, M.G.; Sharov, A.A.; Ko, M.S. Dynamics of global gene expression changes during mouse preimplantation development. *Dev Cell*, 2004, *6*, 117-131.

[19] Ashburner, M.; Ball, C.A.; Blake, J.A.; Botstein, D.; Butler, H.; Cherry, J.M.; Davis, A.P.; Dolinski, K.; Dwight, S.S.; Eppig, J.T.; Harris, M.A.; Hill, D.P.; Issel-Tarver, L.; Kasarskis, A.; Lewis, S.; Matese, J.C.; Richardson, J.E.; Ringwald, M.; Rubin, G.M.; Sherlock, G. Gene ontology: tool for the unification of biology. The gene ontology consortium. *Nat. Genet.*, 2000, *25*, 25-29.

[20] Dahlquist, K.D.; Salomonis, N.; Vranizan, K.; Lawlor, S.C.; Conklin, B.R. GenMAPP, a new tool for viewing and analyzing microarray data on biological pathways. *Nat. Genet.*, 2002, *31*, 19-20.

[21] Doniger, S.W.; Salomonis, N.; Dahlquist, K.D.; Vranizan, K.; Lawlor, S.C.; Conklin, B.R. MAPPFinder: using Gene Ontology and GenMAPP to create a global gene-expression profile from microarray data. *Genome Biol.*, 2003, *4*, R7.

[22] Hamatani, T.; Falco, G.; Carter, M.G.; Akutsu, H.; Stagg, C.A.; Sharov, A.A.; Dudekula, D.B.; VanBuren, V.; Ko, M.S. Age-associated alteration of gene expression patterns in mouse oocytes. *Hum. Mol. Genet.*, 2004, *13*, 2263-2278.

[23] Pan, H.; Ma, P.; Zhu, W.; Schultz, R.M. Age-associated increase in aneuploidy and changes in gene expression in mouse eggs. *Dev. Biol.*, 2008, *316*, 397-407.

[24] Strutz, F.; Okada, H.; Lo, C.W.; Danoff, T.; Carone, R.L.; Tomaszewski, J.E.; Neilson, E.G. Identification and characterization of a fibroblast marker: FSP1. *J. Cell Biol.*, 1995, *130*, 393-405.

[25] Iwano, M.; Plieth, D.; Danoff, T.M.; Xue, C.; Okada, H.; Neilson, E.G. Evidence that fibroblasts derive from epithelium during tissue fibrosis. *J. Clin. Invest.*, 2002, *110*, 341-350.

[26] Janda, E.; Lehmann, K.; Killisch, I.; Jechlinger, M.; Herzig, M.; Downward, J.; Beug, H.; Grunert, S. Ras and TGF[beta] cooperatively regulate epithelial cell plasticity and metastasis: dissection of Ras signaling pathways. *J. Cell Biol.*, 2002, *156*, 299-313.

[27] Kiemer, A.K.; Takeuchi, K.; Quinlan, M.P. Identification of genes involved in epithelial-mesenchymal transition and tumor progression. *Oncogene*, 2001, *20*, 6679-6688.

[28] Vincent-Salomon, A.; Thiery, J.P. Host microenvironment in breast cancer development: epithelial-mesenchymal transition in breast cancer development. *Breast Cancer Res*, 2003, *5*, 101-106.

[29] Xue, C.; Plieth, D.; Venkov, C.; Xu, C.; Neilson, E.G. The gatekeeper effect of epithelial-mesenchymal transition regulates the frequency of breast cancer metastasis. *Cancer Res.*, 2003, *63*, 3386-3394.

[30] Ferrari, G.; Cusella-De Angelis, G.; Coletta, M.; Paolucci, E.; Stornaiuolo, A.; Cossu, G.; Mavilio, F. Muscle regeneration by bone marrow-derived myogenic progenitors. *Science*, 1998, *279*, 1528-1530.

[31] Krause, D.S.; Theise, N.D.; Collector, M.I.; Henegariu, O.; Hwang, S.; Gardner, R.; Neutzel, S.; Sharkis, S.J. Multi-organ, multi-lineage engraftment by a single bone marrow-derived stem cell. *Cell*, 2001, *105*, 369-377.

[32] Petersen, B.E.; Bowen, W.C.; Patrene, K.D.; Mars, W.M.; Sullivan, A.K.; Murase, N.; Boggs, S.S.; Greenberger, J.S.; Goff, J.P. Bone marrow as a potential source of hepatic oval cells. *Science*, 1999, *284*, 1168-1170.

[33] Sanchez-Ramos, J.; Song, S.; Cardozo-Pelaez, F.; Hazzi, C.; Stedeford, T.; Willing, A.; Freeman, T.B.; Saporta, S.; Janssen, W.; Patel, N.; Cooper, D.R.; Sanberg, P.R. Adult bone marrow stromal cells differentiate into neural cells *in vitro*. *Exp. Neurol.*, 2000, *164*, 247-256.

[34] Woodbury, D.; Schwarz, E.J.; Prockop, D.J.; Black, I.B. Adult rat and human bone marrow stromal cells differentiate into neurons. *J. Neurosci. Res.*, 2000, *61*, 364-370.

[35] Kohyama, J.; Abe, H.; Shimazaki, T.; Koizumi, A.; Nakashima, K.; Gojo, S.; Taga, T.; Okano, H.; Hata, J.; Umezawa, A. Brain from bone: efficient "meta-differentiation" of marrow stroma-derived mature osteoblasts to neurons with Noggin or a demethylating agent. *Differentiation*, 2001, *68*, 235-244.

[36] Kim, B.J.; Seo, J.H.; Bubien, J.K.; Oh, Y.S. Differentiation of adult bone marrow stem cells into neuroprogenitor cells *in vitro*. *Neuroreport*, 2002, *13*, 1185-1188.

[37] Solursh, M. Differentiation of cartilage and bone. *Curr. Opin. Cell Biol.*, 1989, *1*, 989-994.

[38] Hauselmann, H.J.; Fernandes, R.J.; Mok, S.S.; Schmid, T.M.; Block, J.A.; Aydelotte, M.B.; Kuettner, K.E.; Thonar, E.J. Phenotypic stability of bovine articular chondrocytes after long-term culture in alginate beads. *J. Cell Sci.*, 1994, *107 (Pt 1)*, 17-27.

[39] Reginato, A.M.; Iozzo, R.V.; Jimenez, S.A. Formation of nodular structures resembling mature articular cartilage in long-term primary cultures of human fetal epiphyseal chondrocytes on a hydrogel substrate. *Arthritis Rheum.*, 1994, *37*, 1338-1349.

[40] Archer, C.W.; McDowell, J.; Bayliss, M.T.; Stephens, M.D.; Bentley, G. Phenotypic modulation in sub-populations of human articular chondrocytes *in vitro*. *J. Cell Sci.*, 1990, *97(Pt 2)*, 361-371.

[41] Benya, P.D.; Padilla, S.R.; Nimni, M.E. Independent regulation of collagen types by chondrocytes during the loss of differentiated function in culture. *Cell*, 1978, *15*, 1313-1321.

[42] Benya, P.D.; Shaffer, J.D. Dedifferentiated chondrocytes reexpress the differentiated collagen phenotype when cultured in agarose gels. *Cell*, 1982, *30*, 215-224.

[43] Lefebvre, V.; Peeters-Joris, C.; Vaes, G. Production of collagens, collagenase and collagenase inhibitor during the dedifferentiation of articular chondrocytes by serial subcultures. *Biochim. Biophys. Acta*, 1990, *1051*, 266-275.

[44] Bonaventure, J.; Kadhom, N.; Cohen-Solal, L.; Ng, K.H.; Bourguignon, J.; Lasselin, C.; Freisinger, P. Reexpression of cartilage-specific genes by dedifferentiated human articular chondrocytes cultured in alginate beads. *Exp. Cell Res.*, 1994, *212*, 97-104.

[45] Yoon, Y.M.; Kim, S.J.; Oh, C.D.; Ju, J.W.; Song, W.K.; Yoo, Y.J.; Huh, T.L.; Chun, J.S. Maintenance of differentiated phenotype of articular chondrocytes by protein kinase C and extracellular signal-regulated protein kinase. *J. Biol. Chem.*, 2002, *277*, 8412-8420.

[46] Matsumoto, S.; Shibuya, I.; Kusakari, S.; Segawa, K.; Uyama, T.; Shimada, A.; Umezawa, A. Membranous osteogenesis system modeled with KUSA-A1 mature osteoblasts. *Biochim. Biophys. Acta*, 2005, *1725*, 57-63.

[47] Umezawa, A.; Maruyama, T.; Segawa, K.; Shadduck, R.K.; Waheed, A.; Hata, J. Multipotent marrow stromal cell line is able to induce hematopoiesis *in vivo*. *J. Cell. Physiol.*, 1992, *151*, 197-205.

[48] Sugiki, T.; Uyama, T.; Toyoda, M.; Morioka, H.; Kume, S.; Miyado, K.; Matsumoto, K.; Saito, H.; Tsumaki, N.; Takahashi, Y.; Toyama, Y.; Umezawa, A. Hyaline cartilage formation and enchondral ossification modeled with KUM5 and OP9 chondroblasts. *J. Cell. Biochem.*, 2007, *100*, 1240-1254.

[49] Nakano, T.; Kodama, H.; Honjo, T. *In vitro* development of primitive and definitive erythrocytes from different precursors. *Science*, 1996, *272*, 722-724.

[50] Kodama, H.; Nose, M.; Niida, S.; Nishikawa, S. Involvement of the c-kit receptor in the adhesion of hematopoietic stem cells to stromal cells. *Exp. Hematol.*, 1994, *22*, 979-984.

[51] Nakano, T.; Kodama, H.; Honjo, T. Generation of lymphohematopoietic cells from embryonic stem cells in culture. *Science*, 1994, *265*, 1098-1101.

[52] Nakano, T. Lymphohematopoietic development from embryonic stem cells *in vitro*. *Semin. Immunol.*, 1995, *7*, 197-203.

[53] Calvi, L.M.; Adams, G.B.; Weibrecht, K.W.; Weber, J.M.; Olson, D.P.; Knight, M.C.; Martin, R.P.; Schipani, E.; Divieti, P.; Bringhurst, F.R.; Milner, L.A.; Kronenberg, H.M.; Scadden, D.T. Osteoblastic cells regulate the haematopoietic stem cell niche. *Nature*, 2003, *425*, 841-846.

[54] Zhang, J.; Niu, C.; Ye, L.; Huang, H.; He, X.; Tong, W.G.; Ross, J.; Haug, J.; Johnson, T.; Feng, J.Q.; Harris, S.; Wiedemann, L.M.; Mishina, Y.; Li, L. Identification of the haematopoietic stem cell niche and control of the niche size. *Nature*, 2003, *425*, 836-841.

[55] Alizadeh, A.A.; Eisen, M.B.; Davis, R.E.; Ma, C.; Lossos, I.S.; Rosenwald, A.; Boldrick, J.C.; Sabet, H.; Tran, T.; Yu, X.; Powell, J.I.; Yang, L.; Marti, G.E.; Moore, T.; Hudson, J., Jr.; Lu, L.; Lewis, D.B.; Tibshirani, R.; Sherlock, G.; Chan, W.C.; Greiner, T.C.; Weisenburger, D.D.; Armitage, J.O.; Warnke, R.; Levy, R.; Wilson, W.; Grever, M.R.; Byrd, J.C.; Botstein, D.; Brown, P.O.; Staudt, L.M. Distinct types of diffuse large B-cell lymphoma identified by gene expression profiling. *Nature*, 2000, *403*, 503-511.

[56] Beer, D.G.; Kardia, S.L.; Huang, C.C.; Giordano, T.J.; Levin, A.M.; Misek, D.E.; Lin, L.; Chen, G.; Gharib, T.G.; Thomas, D.G.; Lizyness, M.L.; Kuick, R.; Hayasaka, S.; Taylor, J.M.; Iannettoni, M.D.; Orringer, M.B.; Hanash, S. Gene-expression profiles predict survival of patients with lung adenocarcinoma. *Nat. Med.*, 2002, *8*, 816-824.

[57] Pomeroy, S.L.; Tamayo, P.; Gaasenbeek, M.; Sturla, L.M.; Angelo, M.; McLaughlin, M.E.; Kim, J.Y.; Goumnerova, L.C.; Black, P.M.; Lau, C.; Allen, J.C.; Zagzag, D.; Olson, J.M.; Curran, T.; Wetmore, C.; Biegel, J.A.; Poggio, T.; Mukherjee, S.; Rifkin, R.; Califano, A.; Stolovitzky, G.; Louis, D.N.; Mesirov, J.P.; Lander, E.S.; Golub, T.R. Prediction of central nervous system embryonal tumour outcome based on gene expression. *Nature*, 2002, *415*, 436-442.

[58] Rosenwald, A.; Wright, G.; Chan, W.C.; Connors, J.M.; Campo, E.; Fisher, R.I.; Gascoyne, R.D.; Muller-Hermelink, H.K.; Smeland, E.B.; Giltnane, J.M.; Hurt, E.M.; Zhao, H.; Averett, L.; Yang, L.; Wilson, W.H.; Jaffe, E.S.; Simon, R.; Klausner, R.D.; Powell, J.; Duffey, P.L.; Longo, D.L.; Greiner, T.C.; Weisenburger, D.D.; Sanger, W.G.; Dave, B.J.; Lynch, J.C.; Vose, J.; Armitage, J.O.; Montserrat, E.; Lopez-Guillermo, A.; Grogan, T.M.; Miller, T.P.; LeBlanc, M.; Ott, G.; Kvaloy, S.; Delabie, J.; Holte, H.; Krajci, P.; Stokke, T.; Staudt, L.M. The use of molecular profiling to predict survival after chemotherapy for diffuse large-B-cell lymphoma. *N. Engl. J. Med.*, 2002, *346*, 1937-1947.

[59] Shipp, M.A.; Ross, K.N.; Tamayo, P.; Weng, A.P.; Kutok, J.L.; Aguiar, R.C.; Gaasenbeek, M.; Angelo, M.; Reich, M.; Pinkus, G.S.; Ray, T.S.; Koval, M.A.; Last, K.W.; Norton, A.; Lister, T.A.; Mesirov, J.; Neuberg, D.S.; Lander, E.S.; Aster, J.C.; Golub, T.R. Diffuse large B-cell lymphoma outcome prediction by gene-expression profiling and supervised machine learning. *Nat. Med.*, 2002, *8*, 68-74.

[60] van 't Veer, L.J.; Dai, H.; van de Vijver, M.J.; He, Y.D.; Hart, A.A.; Mao, M.; Peterse, H.L.; van der Kooy, K.; Marton, M.J.; Witteveen, A.T.; Schreiber, G.J.; Kerkhoven, R.M.; Roberts, C.; Linsley, P.S.; Bernards, R.; Friend, S.H. Gene expression profiling predicts clinical outcome of breast cancer. *Nature*, 2002, *415*, 530-536.

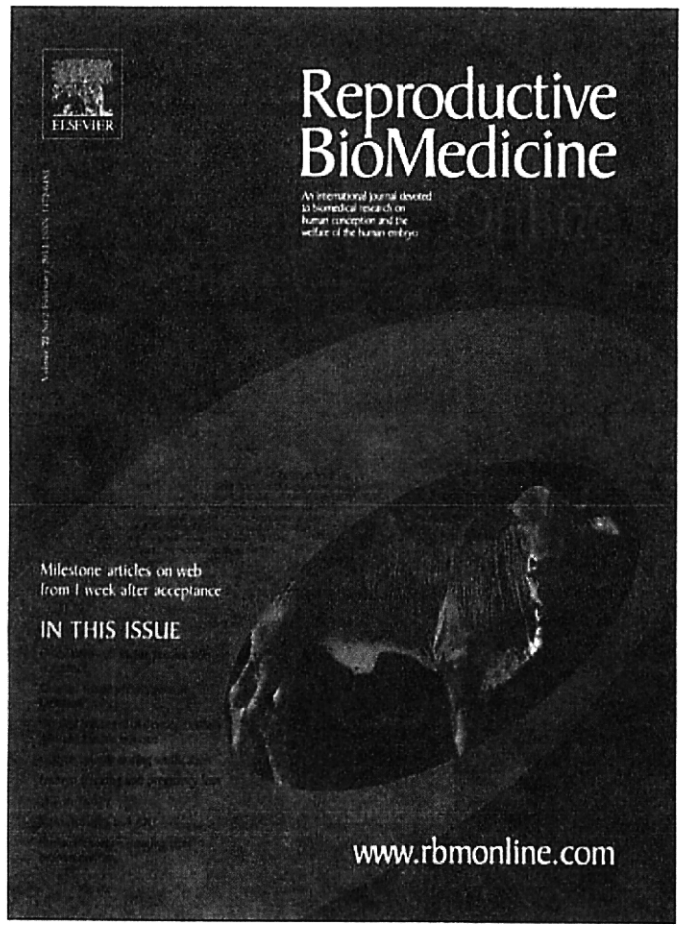
[61] Yeoh, E.J.; Ross, M.E.; Shurtleff, S.A.; Williams, W.K.; Patel, D.; Mahfouz, R.; Behm, F.G.; Raimondi, S.C.; Relling, M.V.; Patel, A.; Cheng, C.; Campana, D.; Wilkins, D.; Zhou, X.; Li, J.; Liu, H.; Pui, C.H.; Evans, W.E.; Naeve, C.; Wong, L.; Downing, J.R. Classification, subtype discovery, and prediction of outcome in pediatric acute lymphoblastic leukemia by gene expression profiling. *Cancer Cell*, 2002, *1*, 133-143.

[62] Scherf, U.; Ross, D.T.; Waltham, M.; Smith, L.H.; Lee, J.K.; Tanabe, L.; Kohn, K.W.; Reinhold, W.C.; Myers, T.G.; Andrews, D.T.; Scudiero, D.A.; Eisen, M.B.; Sausville, E.A.; Pommier, Y.; Botstein, D.; Brown, P.O.; Weinstein, J.N. A gene expression database for the molecular pharmacology of cancer. *Nat. Genet.*, 2000, *24*, 236-244.

[63] Chien, K.R. Stem cells: lost in translation. *Nature*, 2004, *428*, 607-608.

[64] Matoba, R.; Niwa, H.; Masui, S.; Ohtsuka, S.; Carter, M.G.; Sharov, A.A.; Ko, M.S. Dissecting Oct3/4-regulated gene networks in embryonic stem cells by expression profiling. *PLoS One*, 2006, *1*, e26.

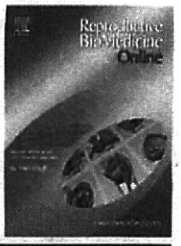
Received: April 07, 2010 Revised: July 09, 2010 Accepted: July 11, 2010



Reproductive BioMedicine Online (2011) 22, 103–105

ELSEVIER

www.sciencedirect.com
www.rbmonline.com



COMMENTARY

Spermatozoal RNA profiling towards a clinical evaluation of sperm quality

Toshio Hamatani

Department of Obstetrics and Gynecology, Keio University School of Medicine, Tokyo, Japan
E-mail address: toshiohamatani@z3.keio.jp

Abstract Human spermatozoal RNAs were recently profiled using microarrays and explored as clinical markers of male infertility. An appropriate study design with a considerable number of biological replicates (sperm samples) is necessary to validate the accuracy and reproducibility of these microarray data. If the genes identified as sperm quality markers by microarray studies are successfully attributed to the pathogenesis of male infertility, then the microarray strategy may be used as a clinical diagnostic tool for male infertility. On the other hand, spermatozoal RNAs may contain not only remnant RNAs after spermatogenesis, but also RNAs that may contribute extragenomically to early embryonic development. Therefore, spermatozoal RNA profiling may enable a better understanding of what is contributed to the oocyte by sperm, in addition to their genome, to facilitate early embryonic development.

KEYWORDS: ICSI, male fertility, RNA profiling, sperm, spermatozoa, transcriptome

Assessments of male reproductive fitness have typically relied upon microscopic evaluation using semen parameters including morphology, motility, sperm concentration, presence of any cell types other than mature spermatozoa, and semen volume. The observation of normal semen features using these parameters does not necessarily guarantee male fertility. Recently, morphological real-time observation at high magnification (> ×6000) has been used to select sperm for intracytoplasmic sperm injection (ICSI). The morphological normalcy of the sperm nucleus is defined by both its shape (smooth, symmetric and oval) and its chromatin content (homogeneity of the chromatin mass containing no extrusion or invagination and no more than one vacuole involving less than 4% of the nuclear area) (Bartoov et al., 2002). Most publications have reported better rates of implantation and clinical pregnancy as well as a reduction in the rate of abortion where sperm cells were strictly morphologically selected at high magnification (Souza Setti et al., 2010). Prospective randomized clinical studies are still needed to confirm the preliminary findings on the efficacy of intracytoplasmic morphologically selected sperm injection (IMSI) over conventional ICSI. The further improvement of diagnosis and treatment of male infertility will need a new method to evaluate sperm quality based on molecular analysis, rather than on morphological observation.

Mature spermatozoa have little cytoplasm and a highly condensed chromatin architecture that is enriched in protamines. These structural features led to the long held view that mature spermatozoa are inert cells but both transcription and translation occur in the mitochondria, and not in the cytoplasm, of mature spermatozoa (Miller and Ostermeier, 2006). Spermatozoal nuclei, containing RNA polymerase and abundant transcription factors, are capable of transcribing RNA from endogenous templates (Hecht and Williams, 1978). Although mature spermatozoa do not contain some of the essential components of the 80S cytoplasmic ribosomes such as 28S and 18S rRNAs; 55S mitochondrial ribosomes are present in spermatozoal polysomal fractions (Gur and Breitbart, 2006). The incorporation of labeled amino acids into polypeptides occurs during sperm

1472-6483/$ - see front matter
doi:10.1016/j.rbmo.2010.12.001

capacitation, and is completely inhibited by mitochondrial translation inhibitors but not by a cytoplasmic translation inhibitor (Gur and Breitbart, 2006). Therefore, it is apparent that mitochondrial ribosomes are actively involved in protein translation in spermatozoa.

The first mRNA that was identified in human mature spermatozoa was the c-Myc mRNA (Kumar et al., 1993). The existence of a complex population of mRNAs in ejaculated human mature spermatozoa was shown by expression profiling using oligo DNA microarrays (Ostermeier et al., 2002). Although these mRNAs were previously thought to be non-functional remnants of stored mRNAs that are synthesized at earlier stages of spermatogenesis, Ostermeier et al. proposed that a specific set of functional RNAs may be delivered into oocytes and support early embryonic development (Ostermeier et al., 2004). Although the specific functional significance of these mRNAs in mature ejaculate spermatozoa remains poorly investigated; they have been demonstrated to influence the phenotypic traits of offspring (Miller and Ostermeier, 2006). The poor developmental ratios relative to normal of both parthenogenetic embryos and cloned embryos obtained from somatic-cell nuclear transfer, are consistent with a developmental role for spermatozoal mRNAs.

Garcia-Herrero et al. used microarrays to investigate spermatozoal RNAs (this issue; Garcia-Herrero et al., 2011). They compared the profile gene of expression in spermatozoa that achieved pregnancy (group P) through an ICSI cycle in an oocyte donation program with the profile of those that did not achieve pregnancy (group NP) (Garcia-Herrero et al., 2011). In order to reduce female infertility as a bias factor, all of the oocytes originated from young female donors. Furthermore, the coupled pairs of women (pregnant and non pregnant) received the oocytes from the same donor. The total number of expressed transcripts detected in fresh sperm samples was 19,229. Of those transcripts, 16,035 (83.4%) were expressed in both groups, P and NP. Among these commonly expressed transcripts, only 44 sequences were overexpressed in group P versus NP and five in group NP versus P. Notably, the 44 differentially-expressed genes in group P included four cathepsins and six metallothioneins. Cathepsins are a family of cysteine proteases and are likely to prevent atrophy of seminiferous tubules and support spermatogenesis to pachytene spermatocytes (Gye and Kim, 2004; Wright et al., 2003). Metallothioneins function as detoxicants to prevent damage of the testes by heavy metals (Kusakabe et al., 2008). An ontology analysis by DAVID (http://david.abcc.ncifcrf.gov/home.jsp) of 1358 exclusively-expressed transcripts in group P, found the term 'embryo development ending in birth or egg hatching' as one of the highest-ranked gene ontology (GO) terms. The exclusively-expressed genes corresponding to this GO term include adducin 1 (*ADD1*), activin A receptor type-II like 1 (*ACVRL1*), androgen receptor (*AR*), and aryl-hydrocarbon receptor nuclear translocator (*ARNT*). All of those genes are potential pregnancy success markers rather than potential fertilization makers. In fact, because ICSI removes the physiological process of sperm entry, spermatozoal fertilization factors may not be necessary for pregnancy after ICSI. These highly expressed RNAs in group P may partially represent spermatozoal extra-genomic components that are required for successful pregnancies. In addition, DAVID also analyzed frozen spermatozoa used in ICSI in the same way, and demonstrated that the RNA profile of frozen spermatozoa was considerably changed by the sperm cryopreservation procedure.

Several other studies have profiled spermatozoal RNAs in clinical samples with the aim of finding a marker RNA or a distinctive expression pattern to represent sperm quality. A cross-platform microarray strategy was used to assess the profile of human spermatozoal transcripts from 13 fertile males who had fathered at least one child compared to those from eight teratozoospermic individuals (Platts et al., 2007). This analysis successfully distinguished between the normal and teratozoospermic groups using unsupervised hierarchical clustering. The teratozoospermic group lacked the RNAs of genes related to the ubiquitin-proteasome pathway and those genes transcribed at late stages of spermatogenesis including; an egg-activating sperm factor, *PLCZ1*; acrosomal proteins, *ACRV1* and *SPAM1*; and non-tubulin components of sperm tails, *ODF1-4*. These changes in gene expression are indicative of the failure of late-stage spermatogenesis in teratozoospermia. In a study by Lalancette et al., the spermatozoal RNAs of 24 healthy donors were expression profiled and a series of invariable transcripts were consistently present in all of the donor samples (Lalancette et al., 2009). Based on the expression of these consistently-expressed genes, only a single donor sample was not well correlated with the other 23 samples, suggesting that spermatozoal RNA profiling could be clinically applied to mark outliers. Furthermore, Garcia-Herrero et al. compared the transcriptomic profiles of sperm samples that achieved pregnancy after the first IUI to those that did not (Garcia-Herrero et al., 2009). They identified 756 genes that were significantly preferentially expressed in the pregnant group, and 194 genes that were significantly preferentially expressed in the non-pregnant group (Garcia-Herrero et al., 2009). Interestingly, these 756 genes include 20 of the 44 genes that were overexpressed in group P (pregnant after an ICSI in oocyte donation program) in the current study by the same group described in this volume of *Reproductive BioMedicine Online*. These 20 genes out of the 756 genes could be considered as potential pregnancy success markers rather than potential fertilization makers.

Thus far, microarray technologies have been used to assess the profiles of human spermatozoal RNAs and the utility of spermatozoal RNAs as clinical markers of male infertility. An appropriate study design with a considerable number of biological replicates (sperm samples) is necessary to validate the accuracy and reproducibility of these microarray data. If the genes identified as sperm quality markers by microarray studies are successfully attributed to the pathogenesis of male infertility, then the microarray strategy may be used as a clinical diagnostic tool for male infertility. On the other hand, spermatozoal RNAs may contain not only RNAs left over from failed or abnormal spermatogenesis, but also RNAs that may contribute extragenomically to early embryonic development. Therefore, spermatozoal RNA profiling may enable a better understanding of what is contributed to the oocyte by sperm, in addition to their genome, to facilitate early embryonic development.

References

Bartoov, B., Berkovitz, A., Eltes, F., et al., 2002. Real-time fine morphology of motile human sperm cells is associated with IVF-ICSI outcome. J. Androl. 23, 1–8.

Garcia-Herrero, S., Garrido, N., Martinez-Conejero, J.A., et al., 2011. Differential transcriptomic profile in spermatozoa achieving pregnancy or not via ICSI. Reprod. BioMed. Online 22, 25–36.

Garcia-Herrero, S., Meseguer, M., Martinez-Conejero, J.A., et al., 2009. The transcriptome of spermatozoa used in homologous intrauterine insemination varies considerably between samples that achieve pregnancy and those that do not. Fertil. Steril. 94, 1360–1373.

Gur, Y., Breitbart, H., 2006. Mammalian sperm translate nuclear-encoded proteins by mitochondrial-type ribosomes. Genes Dev. 20, 411–416.

Gye, M.C., Kim, S.T., 2004. Expression of cathepsin L in human testis under diverse infertility conditions. Arch. Androl. 50, 187–191.

Hecht, N.B., Williams, J.L., 1978. Synthesis of RNA by separated heads and tails from bovine spermatozoa. Biol. Reprod. 19, 573–579.

Kumar, G., Patel, D., Naz, R.K., 1993. C-MYC mRNA is present in human sperm cells. Cell. Mol. Biol. Res. 39, 111–117.

Kusakabe, T., Nakajima, K., Suzuki, K., et al., 2008. The changes of heavy metal and metallothionein distribution in testis induced by cadmium exposure. Biometals 21, 71–81.

Lalancette, C., Platts, A.E., Johnson, G.D., et al., 2009. Identification of human sperm transcripts as candidate markers of male fertility. J. Mol. Med. 87, 735–748.

Miller, D., Ostermeier, G.C., 2006. Towards a better understanding of RNA carriage by ejaculate spermatozoa. Hum. Reprod. Update 12, 757–767.

Ostermeier, G.C., Miller, D., Huntriss, J.D., et al., 2004. Reproductive biology: delivering spermatozoan RNA to the oocyte. Nature 429, 154.

Ostermeier, G.C., Dix, D.J., Miller, D., et al., 2002. Spermatozoal RNA profiles of normal fertile men. Lancet 360, 772–777.

Platts, A.E., Dix, D.J., Chemes, H.E., et al., 2007. Success and failure in human spermatogenesis as revealed by teratozoospermic RNAs. Hum. Mol. Genet. 16, 763–773.

Souza Setti, A., Ferreira, R.C., Paes de Almeida Ferreira Braga, D., et al., 2010. Intracytoplasmic sperm injection outcome versus intracytoplasmic morphologically selected sperm injection outcome: a meta-analysis. Reprod. Biomed. Online 21, 450–455.

Wright, W.W., Smith, L., Kerr, C., et al., 2003. Mice that express enzymatically inactive cathepsin L exhibit abnormal spermatogenesis. Biol. Reprod. 68, 680–687.

Declaration: The authors declare no conflicts of interest.

Received 30 November 2010; accepted 2 December 2010.

High Mobility Group Box 1 (HMGB1) Levels in the Placenta and in Serum in Preeclampsia

Bo Wang*, Kaori Koga, Yutaka Osuga, Tetsuya Hirata, Ako Saito, Osamu Yoshino, Yasushi Hirota, Miyuki Harada, Yuri Takemura, Tomoyuki Fujii, Yuji Taketani

Department of Obstetrics and Gynecology, University of Tokyo, Tokyo, Japan

Keywords
Apoptosis, hypoxia, inflammation, oxidative stress, receptor for advanced glycation end products, toll-like receptors

Correspondence
Kaori Koga, Department of Obstetrics and Gynecology, University of Tokyo, 7-3-1 Hongo Bunkyo, Tokyo 113-8655, Japan.
E-mail: kawotan-tky@umin.ac.jp

*Present address: Obstetrics and Gynecology, Women's Hospital, School of Medicine, Zhejiang University, Zhejiang, China.

Submitted October 4, 2010;
accepted December 13, 2010.

Citation
Wang B, Koga K, Osuga Y, Hirata T, Saito A, Yoshino O, Hirota Y, Harada M, Takemura Y, Fujii T, Taketani Y. High mobility group Box 1 (HMGB1) levels in the placenta and in serum in preeclampsia. Am J Reprod Immunol 2011

doi:10.1111/j.1600-0897.2010.00975.x

Problem
Preeclampsia is a pregnancy disorder characterized by systemic inflammation. High mobility group box 1 (HMGB1) is a molecule known to act as a 'danger signal' by participating in various inflammatory processes, but data in regard to preeclampsia are sparse. The aim of this study was to analyze placental and serum HMGB1 levels in normal pregnancy and preeclampsia.

Method of study
Sera were collected from women with preeclampsia soon after the manifestation of the disease and before commencing any medication. Placental samples were collected immediately after delivery. Expressed isoforms of HMGB1 (28- and 30-kDa) in the placenta were evaluated by Western blot analysis. Serum HMGB1 concentrations were measured using enzyme-linked immunosorbent assays (ELISA).

Results
Two isoforms of HMGB1 are expressed by the human placenta. The 28- and 30-kDa HMGB1 isoforms were expressed highly in preeclamptic placental tissue; however, compared with normotensive control tissue, differences in detected expression levels did not reach statistical significance. No significant difference was observed in serum HMGB1 levels between control and preeclampsia.

Conclusion
Inflammation provoked by HMGB1 is likely to be involved in the proinflammatory process in preeclamptic placenta. Further studies are needed to elucidate the precise role of HMGB1 in preeclampsia.

Introduction

Preeclampsia is a placenta-originated disorder and affects 3–5% of all pregnancies. It remains as one of the leading contributors to maternal and fetal morbidity and mortality.[1] It is a disorder characterized by intravascular inflammation and endothelial cell dysfunction. Despite recent progress in research, the biology of preeclampsia is still poorly understood.[2]

High mobility group box 1 (HMGB1), a non-histone chromatin-associated protein, was discovered three decades ago as a nuclear protein that migrates quickly during electrophoresis and was named according to this property.[3] HMGB1 is released from damaged cells and acts as a 'danger signal' by

participating in various inflammatory processes, including maturation of immune cells, release of cytokines and other inflammatory mediators, and tissue remodeling.[4,5] HMGB1 mediates its inflammatory responses by signaling via receptors such as the receptor for advanced glycation end products (RAGE)[6] and toll-like receptor (TLR) 2 and TLR4.[7] Ligation for these receptors results in activation of nuclear factor kappa B (NFκB), which induces upregulation of leukocyte adhesion molecules and the production of proinflammatory cytokines in both hematopoietic and endothelial cells, thereby promoting inflammation.

It has been demonstrated that HMGB1 is involved in the pathogenesis of a variety of both infectious and non-infectious inflammatory conditions. Elevated levels of HMGB1 in serum and tissues are observed during infection and tissue injury, and targeting HMGB1 with specific antagonists can have protective effects in established inflammatory diseases. For instance, circulating HMGB1 levels are markedly increased during severe sepsis,[8] pneumonia,[9] systemic lupus erythematosis,[10] and in the synovial fluid of patients with rheumatoid arthritis.[11] Administration of HMGB1 antagonists has been reported to decrease organ damage and mortality in models of systemic inflammation such as sepsis,[12,13] brain infarction,[14] arthritis,[15] acute pancreatitis,[16] and lung inflammation.[17]

Preeclampsia is characterized by an inflammatory state that includes elevated levels of proinflammatory molecules in the placenta and maternal serum.[18] The expression of RAGE, one of the receptors for HMGB1, was reported to be significantly higher in preeclamptic placenta when compared with normal placental tissue.[19,20] TLR4, also a receptor for HMGB1, is expressed higher in trophoblasts from patients with preeclampsia compared to normal pregnancies.[21,22] As for HMGB1, Holmlund et al.[23] demonstrated its expression in the trophoblasts by immunohistochemistry. Further immunohistochemical analysis demonstrated higher expression levels of cytoplasmic HMGB1 in the decidua from women with preeclampsia compared with normal pregnancy, but the difference was not conclusive in trophoblasts.[23] The circulating level of HMGB1 in pregnant women has never been elucidated.

In this study, we measured HMGB1 levels in the placenta and serum in normal pregnancies and pregnancies complicated by preeclampsia to ascertain whether this molecule is involved in the pathogenesis of preeclampsia.

Materials and methods

Serum and Tissue Collection

The study was approved by the ethical committee of the University of Tokyo and Musashino Red Cross Hospital, and written informed consent was obtained from all women. Placentas and maternal venous blood were obtained from women with uncomplicated, normotensive pregnancies and pregnancies complicated by preeclampsia. Preeclampsia was diagnosed by the presence of hypertension (an absolute blood pressure ≥140 mmHg systolic and/or 90 mmHg diastolic after 20 weeks of gestation) with proteinuria (≥300 mg/24-hr). Patients with preeclampsia did not have any prior history of hypertension or renal disease. All women in control group did not show clinical or pathological signs of preeclampsia, infections, or any other maternal or placental disease.

Blood samples were collected from women with preeclampsia soon after the manifestation of the disease and before commencing any medication. Sera were separated by centrifugation and stored at −70°C before use. Placental samples were collected immediately after delivery. Placental tissue was taken from the middle part of the placenta to avoid amnion and decidual tissue contamination. All samples were stored at −70°C until assayed.

Western Blot Analysis

Placental tissues were homogenized and then sonicated in lysis buffer [10 mM Tris–HCl, 50 mM NaCl, 2 mM EDTA, 1% Triton X-100, (pH 7.0)] with protease inhibitor cocktail (Roche Diagnostics GmbH, Mannheim, Germany). The protein concentration was determined using a modified Bradford protein assay with bovine serum albumin (Sigma-Aldrich, St Louis, MO, USA) as a standard. Thirty micrograms of protein was separated on 12.5% sodium dodecyl sulfate polyacrylamide electrophoresis gel and then transferred onto polyvinylidene fluoride (PVDF) transfer membranes (Amersham Biosciences, Piscataway, NJ, USA). Protein extracted from human endometrium was used as a positive control.[24] The blots were blocked in tris-buffered saline – 0.1% Tween-20 containing 5% nonfat milk and then incubated with antibodies at 4°C overnight. The membranes were incubated with primary antibodies: anti-human HMGB1 antibody (final concentration 2 μg/mL; R & D

systems, Minneapolis, MN, USA) or goat anti-human actin antibody (1/1000; Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA) as a loading control. Normal mouse IgG2B (Amersham Biosciences, Little Chalfont, UK) was used as an isotype control. The secondary antibody was horseradish peroxidase-conjugated anti-mouse (1/1000; Amersham Biosciences) or anti-goat (1/5000; Santa Cruz) IgG, which was incubated for 1 hr at room temperature. Signals were developed using ECL Western blotting system (Amersham Biosciences). Densitometric analysis was performed using IMAGEJ IMAGE Software (National Institutes of Health, Bethesda, MD, USA). Each HMGB1 band was normalized to the densitometric value obtained from the same lane by blotting for actin, the internal reference.

Enzyme-linked Immunosorbent Assay (ELISA) Measurement of HMGB1

The concentration of HMGB1 in serum was measured in duplicate by a specific ELISA kit (Shino-test Corporation, Kanagawa, Japan). The minimum detectable dose of HMGB1 was 1 ng/mL. The intra- and inter-assay coefficients of variation were all <10%.

Statistical Analysis

Data analysis was performed using the statistical software package SPSS for Windows (Chicago, IL, USA). All data were checked for their normal distribution by submission to the Kolmogorov–Smirnov test, and if significant, non-parametric statistical analysis was applied. Parametric variables underwent the Student's *t*-test. Statistical significance was considered as $P < 0.05$.

Results

We firstly analyzed HMGB1 expression in the placenta. Western blot analysis showed that the human term placenta expresses HMGB1 and is detected as a 28- and 30-kDa band corresponding to two distinct isoforms of the molecule (Fig. 1). The latter band corresponds to biologically active acetylated isoform.[5,24]

We then compared the placental expression levels of the two isoforms between normal pregnancy and pregnancy complicated by preeclampsia. Maternal age, gestational age, parity, and mode of in delivery were comparable in both groups (Table I). Compared

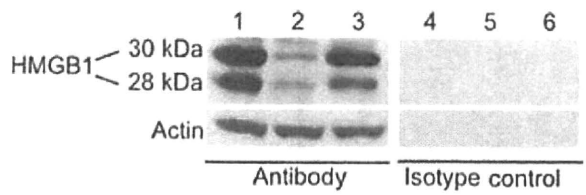


Fig. 1 A representative result of Western blot analysis in the placenta in preeclampsia (1, 4), normal pregnancy (2, 5) and human endometrium as a positive control (3, 6) for anti-high mobility group box 1 (HMGB1) or anti-actin antibody (1, 2, 3) and isotype negative control (4, 5, 6). Note there are two bands (28- and 30-kDa) specific for HMGB1.

Table I Clinical Backgrounds and Serum High Mobility Group Box 1 (HMGB1) Concentrations in Women With or Without Preeclampsia

	Normal pregnancy ($n = 32$)	Preeclampsia ($n = 35$)	P
Maternal age, year*	32.94 ± 3.58	33.49 ± 4.09	NS
Gestational age, week*	34.40 ± 4.90	33.50 ± 4.78	NS
HMGB1, ng/mL			
Median (IQR)	4.757 (2.592–6.861)	4.312 (2.451–6.011)	
Mean ± S.D.	5.119 ± 2.773	4.511 ± 2.537	NS

IQR, interquartile range.
*Data are presented as mean ± S.D.

to normal pregnancy, the level of 28- and 30-kDa HMGB1 expression was higher in preeclampsia, especially in the 28-kDa isoform (normal versus preeclampsia: 0.176 ± 0.112 versus 0.363 ± 0.296, 0.463 ± 0.332 versus 0.581 ± 0.379; 28-, 30-kDa, respectively: mean ± S.D.), although the difference did not reach statistical significance ($P = 0.087$, $P = 0.471$; 28-, 30-kDa, respectively) (Fig. 2, Table II).

Secondly, we measured the level of serum HMGB1 in normal pregnancy and pregnancy complicated with preeclampsia. As shown in Table II, maternal age and gestational age were comparable between the normal and preeclampsia group. When we compared serum HMGB1 concentrations, there was no significant difference between controls and women with preeclampsia.

Fig. 3 shows the correlation between serum HMGB1 concentration and gestational ages for both groups. There was no correlation between gestational

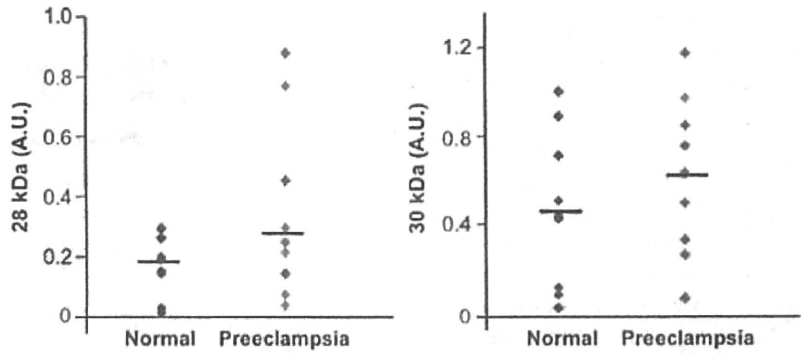


Fig. 2 A scatter plot of placental high mobility group box 1 (HMGB1) protein expression level in both 28- and 30-kDa bands in normal pregnancy and pregnancy complicated with preeclampsia. The data are presented as arbitrary densitometric units (A.U.). The horizontal bars indicate the mean. The expression level of HMGB1 protein in both isoforms was higher in preeclampsia, although the difference did not reach statistical significance ($P = 0.087$, $P = 0.471$; 28-, 30-kDa, respectively).

Table II. Clinical Backgrounds and Placental High Mobility Group Box 1 (HMGB1) Expression Levels in Women With or Without Preeclampsia

	Normal pregnancy ($n = 10$)	Preeclampsia ($n = 10$)	P
Primigravid (n)	7	7	NS[a]
Maternal age, year*	30.00 ± 5.14	33.40 ± 3.69	NS[b]
Gestational age, week*	38.81 ± 1.18	36.20 ± 3.26	NS[b]
Vaginal delivery (n)	7	3	NS[a]
HMGB1 28 kDa (A.U.)*	0.176 ± 0.112	0.363 ± 0.296	NS[b]
HMGB1 30 kDa (A.U.)*	0.463 ± 0.332	0.581 ± 0.379	NS[b]

*Data are presented as mean ± S.D.
[a]Fisher's Exact test.
[b]Student's t-test.

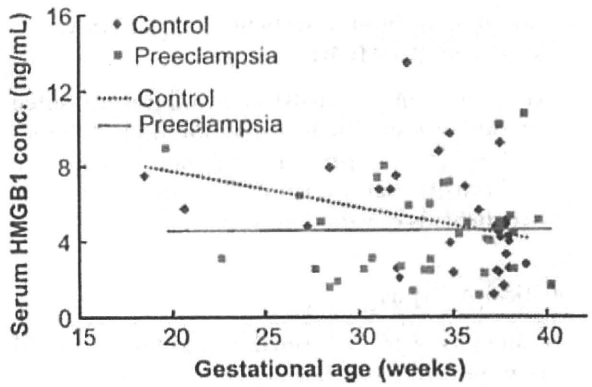


Fig. 3 A scatter plot of serum high mobility group box 1 (HMGB1) levels versus gestational age (weeks) in normal pregnancy (blue diamond dots) and women with preeclampsia (pink square dots). No statistically significant correlation was found between gestational age and serum HMGB1 level in either normal pregnancy (broken line; $r = -0.338$, $P = 0.058$) or women with preeclampsia (unbroken line; $r = 0.002$, $P = 0.993$).

age and serum HMGB1 level in normal pregnancies (Pearson correlation, $r = -0.338$, $P = 0.058$) or in women with preeclampsia ($r = 0.002$, $P = 0.993$).

Discussion

In the present study, we showed that the expression of HMGB1 in the placenta was higher in preeclampsia compared with normal pregnancy, although the difference did not reach statistical significance. There was no difference in serum HMGB1 levels between groups. These findings add to our understanding of the possible involvement of HMGB1 in the pathology of preeclampsia.

Firstly, a quantitative evaluation of HMGB1 expression in the placenta by Western blot demonstrated that the expression of 28- and 30-kDa isotypes in the placenta from women with preeclampsia was higher compared to healthy pregnancies, although the difference did not reach statistical significance. A variety of factors are reported to induce the expression of HMGB1 such as necrosis,[25] apoptosis,[26] oxidative stress,[27] and hypoxia,[15] which are all known to be enhanced in the placenta in preeclampsia. Therefore, we speculate that the expression of HMGB1 is increased in the damaged preeclamptic placenta as a 'danger signal', further enhancing the immune response.

Given the observation that placental HMGB1 is higher in preeclampsia, together with the fact that its receptors, RAGE and TLR4, are upregulated in the placenta in preeclampsia,[19,20,22,28] we suggest that the proinflammatory axis provoked by HMGB1 is enhanced in the preeclamptic placenta. Indeed, changes that may be induced by HMGB1 include NFκB activation, followed by the production of proinflammatory cytokines such as TNF alpha,[29] IL-6,[30] and endothelin,[31] or induction of apoptosis[32,33] are all events observed in the preeclamptic placenta. Although other endogenous and exogenous factors besides HMGB1 may also bind to RAGE and TLRs, such as advanced glycation end products (AGE) to RAGE, lipopolysaccharides and heat-shock protein 70 to TLR4, or peptidoglycan to TLR2, our result suggests that HMGB1 is one of the contributors modulating the development of preeclampsia.

There are several explanations for the lack of significant difference in placental HMGB1 levels between preeclampsia and control. Firstly, the sample number in this study was so small that the statistical study was underpowered. It is also possible that Western blotting followed by densitometry analysis has a limitation in detecting subtle difference. Another explanation could be that even in the healthy condition, the placenta is exposed by a mild inflammation, which is a nature of normal pregnant uterine environment,[34] and HMGB1 is constitutively expressed regardless of whether healthy or preeclamptic condition.

We then measured the circulating levels of HMGB1 in pregnancy. Our observation that placental HMGB1 is slightly higher in preeclampsia, and given a greater amount of trophoblast fragments are detected in the maternal circulation in preeclampsia compared to normal pregnancy,[35] prompted us to hypothesize that the circulating level of HMGB1 is higher in preeclampsia. Contrary to our hypothesis, there was no difference in the serum level of HMGB1 between normal pregnancy and pregnancy complicated by preeclampsia. One explanation could be that the level of circulating HMGB1 does not reflect its release from the placenta. This is partially supported by our finding that serum HMGB1 levels did not positively correlate with gestational age, yet HMGB1 levels should be in proportion to placental size and the number of shedding trophoblasts entering the maternal circulation. It is also possible that some component present in serum may bind HMGB1 and interfere with the ELISA system, as reported by Urbonaviciute et al.[10] Indeed, this interference resulted in an underestimation of serum HMGB1 levels in rheumatoid arthritis.[36] In addition, soluble RAGE (sRAGE), which is reported to capture and eliminate circulating HMGB1,[37] may have affected our results because circulating sRAGE levels are known to be elevated in preeclampsia.[28] Therefore, our results do not exclude the possibility that circulating HMGB1 is elevated in preeclampsia and could be a therapeutic target for preeclampsia.

In summary, we have demonstrated that the levels of HMGB1 in the placenta were slightly higher in preeclampsia. Inflammation provoked by HMGB1 is likely to be involved in the proinflammatory event, which is a prominent feature found in preeclamptic placenta. Further studies are needed to elucidate the precise role of HMGB1 in preeclampsia.

Acknowledgments

The authors thank medical colleagues in the University of Tokyo Hospital, Musashino Red Cross Hospital, for their assistance in sample collection and Dr. Kate Hale for editing the manuscript. BW is a fellow of Japan China Sasakawa Medical Fellow for YO. This work was supported by grants from Ministry of health, Labor and Welfare and Ministry of Education, Culture, Sports, Science and Technology and by grants from Japan China Medical Association (BW), Yamaguchi Endocrine Research Foundation (KK), the Japan Society for the Study of Hypertension in Pregnancy (KK), and Inohana Alumni Association of Chiba University School of Medicine (KK).

References

1 Sibai B, Dekker G, Kupferminc M: Pre-eclampsia. *Lancet* 2005; 365:785–799.
2 Carty DM, Delles C, Dominiczak AF: Novel biomarkers for predicting preeclampsia. *Trends Cardiovasc Med* 2008; 18:186–194.
3 Goodwin GH, Sanders C, Johns EW: A new group of chromatin-associated proteins with a high content of acidic and basic amino acids. *Eur J Biochem* 1973; 38:14–19.
4 Campana L, Bosurgi L, Rovere-Querini P: HMGB1: a two-headed signal regulating tumor progression and immunity. *Curr Opin Immunol* 2008; 20:518–523.
5 Bianchi ME, Manfredi AA: High-mobility group box 1 (HMGB1) protein at the crossroads between innate and adaptive immunity. *Immunol Rev* 2007; 220:35–46.
6 Hori O, Brett J, Slattery T, Cao R, Zhang J, Chen JX, Nagashima M, Lundh ER, Vijay S, Nitecki D, Morser J, Stern D, Schmidt AM: The receptor for advanced glycation end products (RAGE) is a cellular binding site for amphoterin. Mediation of neurite outgrowth and co-expression of rage and amphoterin in the developing nervous system. *J Biol Chem* 1995; 270:25752–25761.

7 Park JS, Gamboni-Robertson F, He Q, Svetkauskaite D, Kim JY, Strassheim D, Sohn JW, Yamada S, Maruyama I, Banerjee A, Ishizaka A, Abraham E: High mobility group box 1 protein interacts with multiple Toll-like receptors. *Am J Physiol Cell Physiol* 2006; 290:C917–C924.

8 Karlsson S, Pettila V, Tenhunen J, Laru-Sompa R, Hynninen M, Ruokonen E: HMGB1 as a predictor of organ dysfunction and outcome in patients with severe sepsis. *Intensive Care Med* 2008; 34:1046–1053.

9 Angus DC, Yang L, Kong L, Kellum JA, Delude RL, Tracey KJ, Weissfeld L: Circulating high-mobility group box 1 (HMGB1) concentrations are elevated in both uncomplicated pneumonia and pneumonia with severe sepsis. *Crit Care Med* 2007; 35:1061–1067.

10 Urbonaviciute V, Furnrohr BG, Weber C, Haslbeck M, Wilhelm S, Herrmann M, Voll RE: Factors masking HMGB1 in human serum and plasma. *J Leukoc Biol* 2007; 81:67–74.

11 Taniguchi N, Kawahara K, Yone K, Hashiguchi T, Yamakuchi M, Goto M, Inoue K, Yamada S, Ijiri K, Matsunaga S, Nakajima T, Komiya S, Maruyama I: High mobility group box chromosomal protein 1 plays a role in the pathogenesis of rheumatoid arthritis as a novel cytokine. *Arthritis Rheum* 2003; 48:971–981.

12 Wang H, Bloom O, Zhang M, Vishnubhakat JM, Ombrellino M, Che J, Frazier A, Yang H, Ivanova S, Borovikova L, Manogue KR, Faist E, Abraham E, Andersson J, Andersson U, Molina PE, Abumrad NN, Sama A, Tracey KJ: HMG-1 as a late mediator of endotoxin lethality in mice. *Science* 1999; 285:248–251.

13 Yang H, Ochani M, Li J, Qiang X, Tanovic M, Harris HE, Susarla SM, Ulloa L, Wang H, DiRaimo R, Czura CJ, Wang H, Roth J, Warren HS, Fink MP, Fenton MJ, Andersson U, Tracey KJ: Reversing established sepsis with antagonists of endogenous high-mobility group box 1. *Proc Natl Acad Sci U S A* 2004; 101:296–301.

14 Liu K, Mori S, Takahashi HK, Tomono Y, Wake H, Kanke T, Sato Y, Hiraga N, Adachi N, Yoshino T, Nishibori M: Anti-high mobility group box 1 monoclonal antibody ameliorates brain infarction induced by transient ischemia in rats. *FASEB J* 2007; 21:3904–3916.

15 Hamada T, Torikai M, Kuwazuru A, Tanaka M, Horai N, Fukuda T, Yamada S, Nagayama S, Hashiguchi K, Sunahara N, Fukuzaki K, Nagata R, Komiya S, Maruyama I, Fukuda T, Abeyama K: Extracellular high mobility group box chromosomal protein 1 is a coupling factor for hypoxia and inflammation in arthritis. *Arthritis Rheum* 2008; 58:2675–2685.

16 Sawa H, Ueda T, Takeyama Y, Yasuda T, Shinzeki M, Nakajima T, Kuroda Y: Blockade of high mobility group box-1 protein attenuates experimental severe acute pancreatitis. *World J Gastroenterol* 2006; 12:7666–7670.

17 Abraham E, Arcaroli J, Carmody A, Wang H, Tracey KJ: HMG-1 as a mediator of acute lung inflammation. *J Immunol* 2000; 165:2950–2954.

18 Schiessl B: Inflammatory response in preeclampsia. *Mol Aspects Med* 2007; 28:210–219.

19 Chekir C, Nakatsuka M, Noguchi S, Konishi H, Kamada Y, Sasaki A, Hao L, Hiramatsu Y: Accumulation of advanced glycation end products in women with preeclampsia: possible involvement of placental oxidative and nitrative stress. *Placenta* 2006; 27:225–233.

20 Cooke CL, Brockelsby JC, Baker PN, Davidge ST: The receptor for advanced glycation end products (RAGE) is elevated in women with preeclampsia. *Hypertens Pregnancy* 2003; 22:173–184.

21 Wang X, Athayde N, Trudinger B: Placental vascular disease and toll-like receptor 4 gene expression. *Am J Obstet Gynecol* 2005; 192:961–966.

22 Kim YM, Romero R, Oh SY, Kim CJ, Kilburn BA, Armant DR, Nien JK, Gomez R, Mazor M, Saito S, Abrahams VM, Mor G: Toll-like receptor 4: a potential link between "danger signals," the innate immune system, and preeclampsia? *Am J Obstet Gynecol* 2005; 193:921–927.

23 Holmlund U, Wahamaa H, Bachmayer N, Bremme K, Sverremark-Ekstrom E, Palmblad K: The novel inflammatory cytokine high mobility group box protein 1 (HMGB1) is expressed by human term placenta. *Immunology* 2007; 122:430–437.

24 Zicari A, Centonze C, Realacci M, Buchetti B, Pietropolli A, Ticconi C: Estradiol 17-beta and progesterone modulate inducible nitric oxide synthase and high mobility group box 1 expression in human endometrium. *Reprod Sci* 2008; 15:559–566.

25 Scaffidi P, Misteli T, Bianchi ME: Release of chromatin protein HMGB1 by necrotic cells triggers inflammation. *Nature* 2002; 418:191–195.

26 Gauley J, Pisetsky DS: The translocation of HMGB1 during cell activation and cell death. *Autoimmunity* 2009; 42:299–301.

27 Tsung A, Klune JR, Zhang X, Jeyabalan G, Cao Z, Peng X, Stolz DB, Geller DA, Rosengart MR, Billiar TR: HMGB1 release induced by liver ischemia involves Toll-like receptor 4 dependent reactive oxygen species production and calcium-mediated signaling. *J Exp Med* 2007; 204:2913–2923.

28 Germanova A, Koucky M, Hajek Z, Parizek A, Zima T, Kalousova M: Soluble receptor for advanced glycation end products in physiological and pathological pregnancy. *Clin Biochem* 2010; 43:442–446.

29 Pijnenborg R, McLaughlin PJ, Vercruysse L, Hanssens M, Johnson PM, Keith JC Jr, Van Assche FA: Immunolocalization of tumour necrosis factor-alpha (TNF-alpha) in the placental bed of normotensive and hypertensive human pregnancies. *Placenta* 1998; 19:231–239.

30 Lockwood CJ, Yen CF, Basar M, Kayisli UA, Martel M, Buhimschi I, Buhimschi C, Huang SJ, Krikun G, Schatz F: Preeclampsia-related inflammatory cytokines regulate interleukin-6 expression in human decidual cells. *Am J Pathol* 2008; 172:1571–1579.

31 Fiore G, Florio P, Micheli L, Nencini C, Rossi M, Cerretani D, Ambrosini G, Giorgi G, Petraglia F: Endothelin-1 triggers placental oxidative stress pathways: putative role in preeclampsia. *J Clin Endocrinol Metab* 2005; 90:4205–4210.

32 Whitley GS, Dash PR, Ayling LJ, Prefumo F, Thilaganathan B, Cartwright JE: Increased apoptosis in first trimester extravillous trophoblasts from pregnancies at higher risk of developing preeclampsia. *Am J Pathol* 2007; 170:1903–1909.

33 Crocker IP, Cooper S, Ong SC, Baker PN: Differences in apoptotic susceptibility of cytotrophoblasts and syncytiotrophoblasts in normal pregnancy to those complicated with preeclampsia and intrauterine growth restriction. *Am J Pathol* 2003; 162:637–643.

34 Mor G: Inflammation and pregnancy: the role of toll-like receptors in trophoblast-immune interaction. *Ann N Y Acad Sci* 2008; 1127:121–128.

35 Johansen M, Redman CW, Wilkins T, Sargent IL: Trophoblast deportation in human pregnancy – its relevance for pre-eclampsia. *Placenta* 1999; 20:531–539.

36 Voll RE, Urbonaviciute V, Herrmann M, Kalden JR: High mobility group box 1 in the pathogenesis of inflammatory and autoimmune diseases. *Isr Med Assoc J* 2008; 10:26–28.

37 Fukami A, Adachi H, Yamagishi S, Matsui T, Ueda S, Nakamura K, Enomoto M, Otsuka M, Kumagae S, Nanjo Y, Kumagai E, Esaki E, Murayama K, Hirai Y, Imaizumi T: Factors associated with serum high mobility group box 1 (HMGB1) levels in a general population. *Metabolism* 2009; 58:1688–1693.

Bone Morphogenetic Protein-2 (BMP-2) Increases Gene Expression of FSH Receptor and Aromatase and Decreases Gene Expression of LH Receptor and StAR in Human Granulosa Cells

Jia Shi[1], Osamu Yoshino[1,2], Yutaka Osuga[1], Kaori Koga[1], Yasushi Hirota[1], Emi Nose[1], Osamu Nishii[2], Tetsu Yano[1], Yuji Taketani[1]

[1]Department of Obstetrics and Gynecology, University of Tokyo, Tokyo, Japan;
[2]Department of Obstetrics and Gynecology, Mizonokuchi Hospital, Teikyo University, Kawasaki, Japan

Keywords
BMP, folliculogenesis, FSH receptor, HCG, LH receptor, luteinization, ovary

Correspondence
Osamu Yoshino and Yutaka Osuga
Department of Obstetrics and Gynecology, University of Tokyo, Tokyo 113-8655, Japan
E-mail: oyoshino624@hotmail.co.jp and yutakaos-tky@umin.ac.jp

Submitted May 19, 2010;
accepted July 29, 2010.

Citation
Shi J, Yoshino O, Osuga Y, Koga K, Hirota Y, Nose E, Nishii O, Yano T, Taketani Y. Bone morphogenetic protein-2 (BMP-2) increases gene expression of FSH receptor and aromatase and decreases gene expression of LH receptor and stAR in human granulosa Cells. Am J Reprod Immunol 2011; 65: 421–427

doi:10.1111/j.1600-0897.2010.00917.x

Problem
A growing body of evidence indicates that bone morphogenetic protein (BMP) cytokines play a key role in female fertility in mammals. BMP-2 is known to be expressed in the ovary of many species. In the present study, we examined the expression and function of BMP-2 in the human ovary.

Method of Study
BMP-2 mRNA expression in the human ovary was evaluated by *in situ* hybridization. Human granulosa cells were obtained from *in vitro* fertilization patients. Human granulosa cells were cultured with recombinant BMP-2 or human chorionic gonadotrophin (HCG), followed by RNA extraction.

Results
BMP-2 expression was detected in granulosa cells of antral follicles but not of corpus luteum. The *in vitro* study showed that BMP-2 induced follicular stimulating hormone (FSH) receptor and aromatase expression, while decreasing luteinizing hormone (LH) receptor and steroidogenic acute regulatory protein expression in human granulosa cells. HCG decreased gene expression of BMP-2 and increased BMP and activin membrane-bound inhibitor (BAMBI), an antagonist of BMP-2.

Conclusion
Expression and disappearance of BMP-2 might contribute to folliculogenesis and luteinization by regulating gonadotropin receptor expression in human granulosa cells. HCG can modulate BMP-2 function by controlling BMP-2 and BAMBI expression.

Introduction

Proper transition from follicular growth to luteinization is an essential event in the ovulatory follicle for an achievement of pregnancy. In a phase of follicular growth, granulosa cells (GC) express follicular stimulating hormone (FSH) receptor and proliferate under the stimulation of FSH.[1] When the follicle approaches ovulation, the luteinizing hormone (LH) receptor is increasingly expressed in GC. The increased LH

receptor makes GC sensitive to LH surge that cues luteinization of these cells. Progesterone, produced by luteinized GC, sustains the initiation and maintenance of pregnancy. Any perturbation of these events, such as premature luteinization and luteinization failure, can impair reproduction.[2,3]

A growing body of evidence indicates that bone morphogenetic protein (BMP) cytokines, members of the TGF-ß superfamily, play a key role in female fertility in mammals.[4,5] Each molecule shows a spatiotemporally different expression and a specific effect in the ovary. For example, we have reported that BMP-7 and BMP-6 are expressed in theca cells and GC, respectively, in the human ovary, and both substances increase FSH receptor and decrease LH receptor expression in GC.[6,7] Others have demonstrated that BMP-15 and GDF-9 are expressed in oocytes and that mutation of these genes leads to folliculogenesis arrest in human.[8]

Given the importance of BMP cytokines in the human ovary, comprehensive studies of BMP family are needed to understand ovarian physiology and pathology. Other than BMP cytokines described earlier, BMP-2 is also thought to play a role in the ovary. In rat, BMP-2 is expressed in GC, and its expression decreases after ovulation.[4] BMP-2 suppresses progesterone production of GC in sheep[9] and rat,[10] and human granulosa cell-like tumor cell line, KGN cell.[11] According to these findings, we hypothesized that BMP-2 might play a role in regulating luteinization.

To address the roles of BMP-2 in follicular growth and luteinization in the human ovary, we studied its expression and its effect on the expression of FSH receptor, LH receptor, aromatase, and steroidogenic acute regulatory protein (StAR) in human GC. To understand the function of BMP-2, we also studied the expression of BMP and activin membrane-bound inhibitor (BAMBI), a molecule that resembles the type-I receptor and interferes with BMP-2.

Materials and methods

Reagents and Materials

Hyaluronidase, fetal bovine serum (FBS), Dulbecco's minimum essential medium (DMEM)/Ham's F12 (F12) and antibiotics (mixture of penicillin, streptomycin, and amphotericin B) were purchased from Sigma (St. Louis, MO, USA). Recombinant human BMP-2 was purchased from R&D Systems (Minneapolis, MN, USA). Human chorionic gonadotrophin (HCG) was purchased from Mochida (Tokyo, Japan).

Collection of Ovarian Tissues and *In situ* Hybridization for BMP-2

Tissue specimens of human ovaries were obtained under signed informed consent from seven women (age range, 28–40 years old) who underwent salpingo-oophorectomy for a treatment of uterine cervical cancer. All patients had normal ovarian cycles prior to the surgery, and no histological abnormality was observed in ovarian tissues. Among seven patients, four were in follicular phase and three were in luteal phase. The experimental procedure was approved by the institutional review board. Ovarian tissues were fixed in neutral-buffered formalin and embedded in paraffin blocks. *In situ* hybridization was performed using an ISHR Starting kit (Nippon Gene, Toyama, Japan) according to the manufacturer's instructions. To prepare the digoxigenin (DIG)-labeled RNA anti-sense probes for BMP-2, a primer set (NM_001200: 1022–1041 and 1298–1279) was used. Sense probe hybridization was used as a control for a background level.

Cell Culture of Human Luteinized GC

The method to obtain and culture human luteinized GC was described previously.[6,12] Briefly, follicular fluids with luteinized GC were aspirated from patients undergoing oocyte retrieval for *in vitro* fertilization (IVF). The clinical indications for IVF in these patients were primarily male factor or tubal factor infertility. Patients with ovarian dysfunction were excluded from the study. The experimental procedures were approved by the institutional review board, and signed informed consent for use of GC was obtained from each patient. All of the follicular aspirates from each patient were mixed and centrifuged at $200 \times g$ for 5 min, resuspended in PBS with 0.2% hyaluronidase, and incubated at 37°C for 30 min. The suspension was layered onto Ficoll-Paque and centrifuged at $150 \times g$ for 20 min. The GC were collected from the interphase, washed with PBS, and cultured in DMEM/F12 media supplemented with 5% FBS and antibiotics (100 U/mL penicillin, 0.1 mg/mL streptomycin, and 250 ng/mL amphotericin B) for 15 min at 37°C, to remove contaminating macrophage cells from GC. Using this method, GC were remained in the supernatant while macrophages were attached to

American Journal of Reproductive Immunology 65 (2011) 421–427

the culture dish. The collected GC were cultured in DMEM/F12 containing 5% FBS and antibiotics in 12-well plates at a density of 2×10^5 cells/mL and kept at 37°C in a humidified 5% CO_2/95% air environment for 5 days. All of the GC used for the experiments were precultured for 5 days prior to treatments to allow the GC to regain sensitivity to FSH stimulation.[13] Media were changed at 48 -hr intervals. To evaluate the effect of BMP-2, human GC were cultured with or without BMP-2 (100 ng/mL) for 24 hr. To investigate the effect of HCG, GC were cultured with HCG (10 IU/mL) for up to 48 hr. Recombinant BMP-2 was dissolved in 0.1% BSA + 4 mM HCl as a vehicle. The same amount of vehicle was used for a control.

Reverse Transcription and Quantitative Real-time PCR Analysis

Total RNA was extracted from GC, using the RNA-easy minikit (Qiagen, Hilden, Germany). Reverse transcription was performed using Rever Tra Dash (TOYOBO, Tokyo, Japan). One microgram of total RNA was reverse transcribed in a 20-μL volume. For

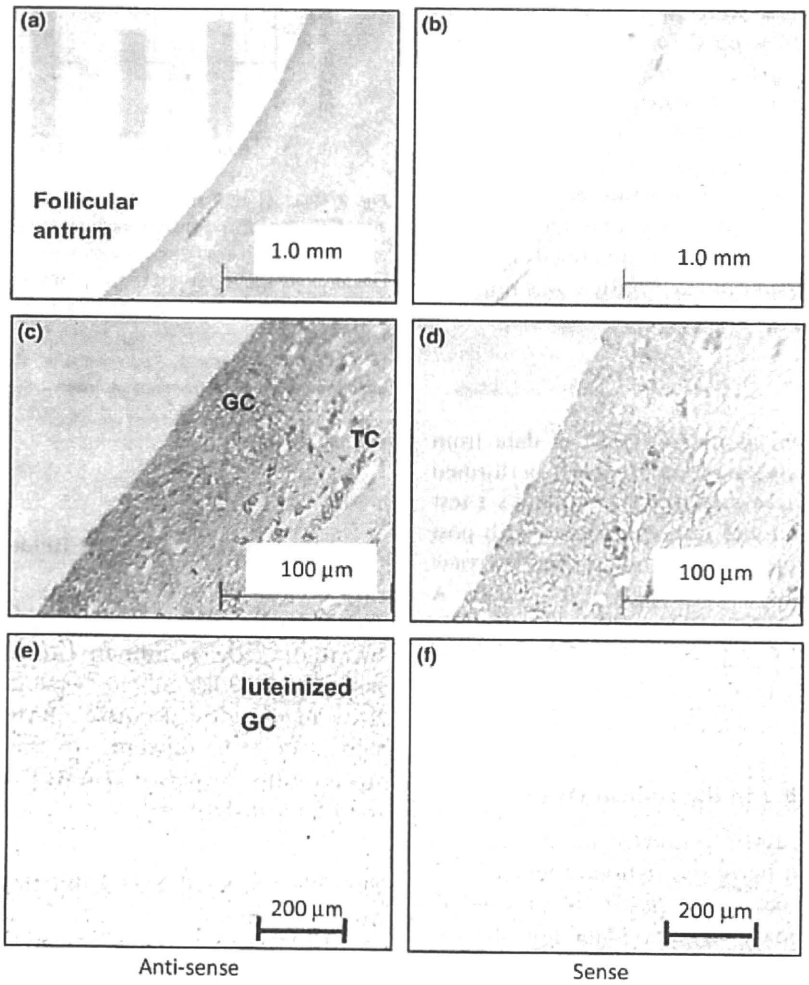


Fig. 1 *In situ* hybridization of bone morphogenetic protein-2 (BMP-2) mRNA of human ovary. Normal human ovaries were examined for BMP-2 mRNA expression. Ovarian tissues were fixed in neutral-buffered formalin and embedded in paraffin blocks. *In situ* hybridization was performed using the digoxigenin (DIG)-labeled RNA anti-sense probes for BMP-2 or sense probe for negative control. Representative data from seven specimens were shown. (a–d): antral follicle, the follicular size was 10 mm in diameter (e and f): corpus luteum; (a, c, and e): BMP-2 anti-sense probe, (b, d, and f): sense control; (a and b): lower magnification (×40), (c–f): higher magnification (×200). GC: granulosa cell layer, TC: theca cell layer.

the quantification of various mRNA levels, real-time PCR was performed using LightCycler (Roche Diagnostic GmbH, Mannheim, Germany), according to the manufacturer's instructions. PCR primer sets were designed to span intron to discriminate PCR products that might arise from possible chromosomal DNA contaminants. The primer sequences were as follows, BMP-2 (NM_001200: 1022–1041 and 1298–1279), BAMBI (NM_012342: 711–730 and 984–965), StAR (NM_000349: 171–190 and 551–532), FSH receptor (NM_000145: 174–196 and 510–492), LH receptor (NM_000233:747–767 and 981–962), and aromatase (NM_031226: 1864–1883 and 2105–2086), GAPDH (NM_002046: 628–648 and 1079–1060). PCR conditions were as follows, BMP-2: 43 cycles of 95°C for 10 s, 65°C for 10 s, and 72°C for 12 s, BAMBI: 31 cycles of 95°C for 10 s, 60°C for 10 s, and 72°C for 11 s, StAR: 35 cycles of 95°C for 10 s, 55°C for 10 s, and 72°C for 15 s, aromatase: 30 cycles of 95°C for 10 s, 62°C for 10 s, and 72°C for 10 s. After amplification, melting curve analysis was followed. PCR conditions for FSH receptor and LH receptor and GAPDH were described in elsewhere.[6,7] The expression of each mRNA was normalized by GAPDH mRNA.

Statistical Analysis

All results are shown as mean ± SEM of data from at least three separate experiments, each performed in triplicate. Data were analyzed by Student's t-test for paired comparison and one-way ANOVA with post hoc test for multiple comparisons using STATVIEW software (SAS Institute Inc., Cary, NC, USA). A *P*-value of less than 0.05 was considered statistically significant.

Results

Localization of BMP-2 in the Human Ovary

The expression of BMP-2 mRNA in the human ovary was examined by *in situ* hybridization. BMP-2 mRNA was barely detectable in GC of primordial, primary, and secondary follicles (data not shown) but detected in GC of antral follicles. Follicular size of antral follicles was from 2.5 mm to 10 mm in diameter. The representative data of an antral follicle with 10 mm in diameter were shown (Fig. 1a–d). In the corpus luteum, BMP-2 mRNA expression in GC was extremely low (Fig. 1e–f).

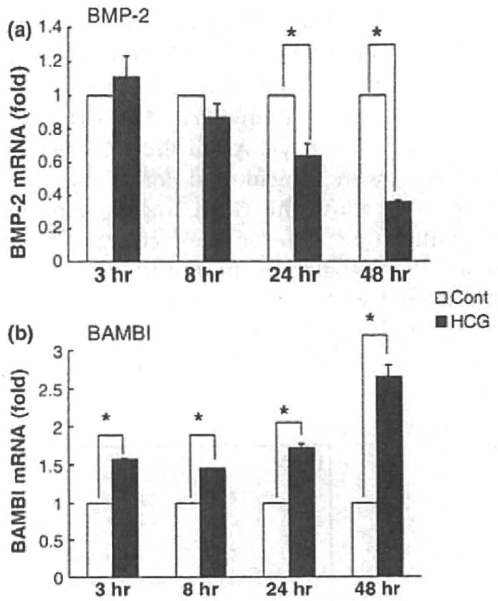


Fig. 2 Effect of HCG on bone morphogenetic protein-2 (BMP-2) (a), and BMP and activin membrane-bound inhibitor (BAMBI) (b) mRNA expression. The human granulosa cells (GC) were cultured with human chorionic gonadotrophin (HCG, 10 IU/mL) for 3–48 hr. Total RNA was extracted from the GC and subjected to real-time polymerase chain reaction (PCR) to determine the mRNA levels. Data were normalized to GAPDH mRNA levels. Representative data from three different experiments were represented as mean ± SEM relative to an adjusted value of 1.0 for the mean value of the each control. *$P < 0.05$ (versus control).

The Effect of HCG on BMP-Related Molecules in GC

To investigate the effect of HCG on BMP-2 and BAMBI mRNA in human GC, cells were cultured with HCG (10 IU/mL) for 3–48 hr (Fig. 2). Notably, HCG significantly decreased BMP-2 mRNA expression from 24 hr onward. On the other hand, HCG significantly increased BAMBI mRNA expression from 3 hr onward.

The Effect of BMP-2 on Folliculogenesis-Related Molecules in GC

In human GC, BMP-2 (100 ng/mL) significantly increased FSH receptor and aromatase mRNA expression (Fig. 3a,e), while BMP-2 significantly decreased LH receptor and StAR mRNA expression (Fig. 3b,c). BMP-2 decreased the expression of StAR in a dose-dependent manner (Fig. 3d)

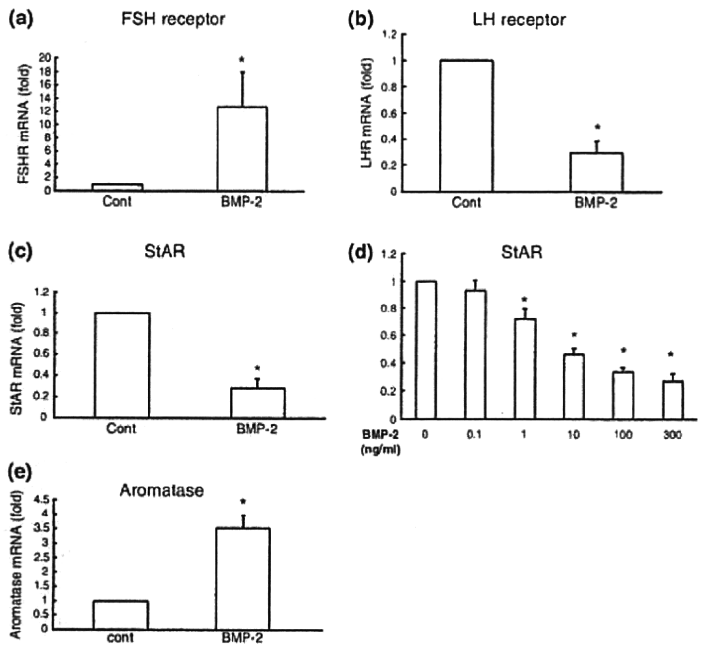


Fig. 3 Effect of bone morphogenetic protein-2 (BMP-2) on FSH receptor (a), LH receptor (b), StAR (c, d) and aromatase (e) mRNA expression. The human granulosa cells (GC) were cultured with BMP-2 (a–c, e: 100 ng/mL, d: 0–300 ng/mL) for 24 hr. Total RNA was extracted from the GC and subjected to real-time polymerase chain reaction (PCR) to determine the mRNA levels. Data were normalized to GAPDH mRNA levels. Data from three different experiments were combined and represented as the mean ± SEM relative to an adjusted value of 1.0 for the mean value of the each control. *$P < 0.05$ (versus control).

(0–300 ng/mL). The dose-dependent changes by BMP-2 were also observed in FSH receptor, aromatase, and LH receptor expression (data not shown).

Discussion

In the present study, we found that BMP-2 mRNA was expressed in GC of antral follicles whereas its expression was almost undetectable in the corpus luteum. In the functional analysis using human GC, HCG decreased BMP-2 mRNA but increased BAMBI mRNA. BMP-2 up-regulated FSH receptor and aromatase mRNA, whereas it down-regulated LH receptor and StAR mRNA.

LH receptor is a key factor in the ability of GC to undergo luteinization.[14,15] StAR, which mediates translocation of cholesterol from the outer to the inner mitochondrial membrane, is one of the rate-limiting factors in progesterone production.[16] Accordingly, the present finding that BMP-2 suppresses the gene expression of these molecules indicates that BMP-2 may play a role as an anti-luteinization factor in the ovary. In contrast, the finding that BMP-2 up-regulated FSH receptor and aromatase gene expression suggests that it promotes follicular growth. Given the expression of BMP-2 exclusively seen in antral follicles, it is plausible that BMP-2 promotes follicular growth and prevents precocious luteinization by regulating the expression of these molecules in GC. On the other hand, the vanishing of BMP-2 expression in the corpus luteum would facilitate luteinization.

In view of BMP-2 effects on GC shown in the present study, the regulation of BMP-2 expression in GC seems to have an impact on the appropriate transition from the growing follicle to the corpus luteum. Our finding that HCG suppressed BMP-2 expression in cultured GC suggests that BMP-2 expression might be attenuated in GC in response to the LH surge *in vivo*. The remarkable decrease in BMP-2 expression in the corpus luteum is consistent with this finding. In this context, it should also be noted that HCG induced expression of BAMBI in GC. BAMBI has a structural feature that resembles type-I receptors but lacks the intracellular serine/threonine kinase domain. BAMBI can compete with