2009). The deregulation of the expression of miRNAs has been shown to contribute to cancer development through various kinds of mechanisms, including deletions, amplifications, or mutations involving miRNA loci, epigenetic silencing, the dysregulation of transcription factors that target specific miRNAs, or the inhibition of processing (Kim et al. 2009).

The expression of microRNA-145 (miR-145), for example, is reported to be low in self-renewing human embryonic stem cells (hESCs) but highly upregulated during differentiation (Xu et al. 2009). Increased miR-145 expression inhibits hESC self-renewal, represses expression of pluripotency genes, and induces lineage-restricted differentiation. On the other hand, miR-145 is reported to be downregulated and to act as a tumor suppressor in various tumors (Chiyomaru et al. 2010). In the same chromosomal region of miR-145, miR-143 is found and reported to act as a tumor suppressor function in the lung metastasis of osteosarcoma (Osaki et al. 2011).

miRNA is also reported to regulate the differentiation of MSC. The role of miRNA in the osteogenic differentiation of MSCs has been indicated by several studies. MiR-125b, 206, and 26a acted as negative regulators of osteoblast differentiation of bone marrow-derived MSCs through negative regulation of ERBB2, Connexin 43, and SMAD1, respectively (reviewed in Guo et al. 2011). MiR-133 and -135 functionally inhibited the differentiation of osteoprogenitors by attenuating RUNX2 and SMAD5 pathways that synergistically contribute to bone formation. On the other hand, miR-196a, -210, and -2861 were shown to enhance osteogenic differentiation, possibly through its target genes *HOXC8*, *ACVR1b*, and *HDAC5*, respectively. Five miRNAs (miR-130b, -152, -28, -26b, and -193b) were found to be differentially expressed during chondrogenic differentiation of MSCs through microarray analysis, and four of them were confirmed by real-time polymerase chain reaction analysis. Furthermore, in the multipotent MSC line C2C12, miR-1, miR-206, and miR-133 exhibited opposing roles in modulating skeletal muscle proliferation and differentiation (reviewed in Guo et al. 2011). These miRNAs might regulate the phenotype of CSCs of sarcoma.

As for the regulation of the CSCs in sarcoma, several studies have emerged. High-throughput microRNA (miRNA) expression analysis revealed that the expression of miR-140 was associated with chemosensitivity in osteosarcoma tumor xenografts (Song et al. 2009). Tumor cells ectopically transfected with miR-140 were more resistant to MTX and 5-fluorouracil (5-FU). Overexpression of miR-140 inhibited cell proliferation in both osteosarcoma U-2 OS (wt-p53) and colon cancer HCT 116 (wt-p53) cell lines. Furthermore, the expression of endogenous miR-140 was significantly elevated in $CD133^+/CD44^+$ colon CSCs that exhibit a slow proliferation rate and strong chemoresistance. However, the effect of miR-140 on CSC of osteosarcoma has not been clarified.

Riggi et al. found that the EWS-FLI-1 fusion gene induces expression of stem cell genes *Oct-4, SOX2,* and *Nanog* in human pediatric MSCs (hpMSCs). hpMSCs expressing EWS-FLI-1 generate a cell subpopulation displaying ESFT CSC features *in vitro.* Their further analysis demonstrated that induction of the ESFT CSC phenotype is the result of the combined effect of EWS-FLI-1 on its target gene expression and repression of miR-145 promoter activity. Subsequently, EWS-FLI-1 and miR-145 proved to function in a mutually repressive feedback loop and identify their common target gene, SOX2, in addition to miR-145 itself, as key players in ESFT cell differentiation and tumorigenicity (Riggi et al. 2010).

Others. Li et al. demonstrated that diallyltrisulfide (DATS), a volatile organosulfur compound derived from garlic, could confer drug resistance of osteosarcoma by downregulation of P-glycoprotein. Their previous report had showed $CD133^+$ SaOS2 cells over expressed MDR1 (P-gp) in comparison with $CD133^-$ SaOS2 cells. The ratio of $CD133^+$ cells in flow cytometric analysis showed no significant difference (around 5.0%) under 0, 0.001, 0.01, 0.1, and 1 ppc of MTX without DATS. However, the ratio decreased (5.4, 3.2, 2.7, 1.4, and 0.92%, respectively) with the same concentration range of MTX in combination with 10μM DATS (Li et al. 2009).

Niche. The concept of "niche" defined as a specialized microenvironment that can promote the emergence of tumor stem cells and provide all the required factors for their development has recently emerged in the literature. Whereas the role of the niche in maintaining stem cells has been shown in several systems, the sarcoma CSC niche has not been identified. However, if sarcoma CSCs arise from MSCs or NCSCs, it is presumable that they might reside within the proposed niche. Several studies suggest that MSCs reside in a perivascular niche in almost all adult tissues, where they are in close contact with blood vessels (Shi and Gronthos 2003, da Silva Meirelles et al. 2008). The location of MSCs within perivascular niches is proposed to support the migration of MSCs in response to injury or disease (Kuhn and Tuan 2010). Similarly, location within a perivascular niche may support the metastasis of sarcomas.

There are some implications that niche plays a certain role in sarcoma CSCs (Siclari and Qin 2010). First, the differences in behavior of sarcoma CSCs grown *in vitro* compared to *in vivo* have been observed. For example, whereas the CSCs are characterized by being quiescent *in vivo,* they are more proliferative than the non-CSCs *in vitro* (Gibbs et al. 2005, Iwasaki et al. 2009). Secondly, especially with relation to bone sarcomas, the bone tissue is a hypoxic environment (Heddeleston et al. 2010, Siclari and Qin 2010). Activation of the hypoxia signaling pathway activates many pathways important for stem cell and CSC maintenance, such as brain CSC, which includes HIF-1α and HIF-2α (Heddeleston et al. 2010). In osteosarcoma,

studies have revealed that the expression of hypoxia-relevant genes occurs mainly in necrotic areas, which suggests that there is an *in vivo* relationship between HIF-1α and apoptosis (Ishenko et al. 2008). In the cases of chondrosarcoma, HIF-1α protein might be a useful prognostic marker due to its important role in tumor angiogenesis and cellular proliferation (Kubo et al. 2008). In Ewing sarcoma, Aryee et al. found that the EWS-FLI1 protein was upregulated in a HIF-1α-dependent manner and that HIF-1α induced EWS-FLI1 accumulation in a time-dependent dynamic study (Aryee et al. 2010). Thirdly, there are some reports on morphological implications. David et al. presented "bone niche" in chondrosarcoma. Pathological findings of chondrosarcoma include infiltration of chondrosarcoma cells into the bone tissue (permeation) and chondrosarcoma development associated with bone resorption foci. They assume that chondrosarcoma growth is strongly linked to bone tissue (David et al. 2011). Indeed, permeation of bony trabeculae is considered an absolute sign of chondrosarcoma and is useful in differentiating Grade 1 chondrosarcoma (malignant) from enchondroma (benign).

These findings support the concept of a niche; however, there is no evidence identifying the role of a niche in sarcoma CSC biology, which had been anticipated.

Immunotherapy

Many clinical trials of CTL-based immunotherapy using peptide vaccination have demonstrated the potency of this new therapeutic modality for various cancers that are resistant to standard chemotherapy (Tsukahara et al. 2004). However, it remains unknown whether CTL-based immunotherapy can kill CSC. Kano et al. found that the SP cells showed the expression of the human leukocyte antigen (HLA) Class I molecules on the cell surface. The CTL clone Tc4C-6, induced by mixed lymphocyte tumor cell culture using autologous peripheral blood mononuclear cells and freshly isolated SP cells, showed specific cytotoxicity against the SP cells. Moreover, the cytotoxicity against SP cells was blocked by the anti-HLA Class I antibody W6/32. These results indicate that CTL-recognizing CSCs certainly exist in the tumor microenvironment and circulating peripheral blood and that SP cells can be killed by CTL. Therefore, CTL-based immunotherapy could be one of the options for targeting sarcoma CSCs.

Conclusions and Perspectives

A great deal of effort has been made in the research on not only the origin of sarcomas but also cancer stem cells of sarcomas. Considering the characteristics of sarcoma CSCs, such as their self-renewal, resistance to

systemic therapy, and high migratory ability, sarcoma CSCs seem to have a lot of similarities with normal tissue stem cells, such as MSCs and NCSCs. However, the relationship between tissue stem cells and cancer stem cells remains unclear. This might be because of the problems categorized below.

The first problem is on the ambiguity of the definitions of cancer stem cells and mesenchymal stem cells. These precise definitions remain to be developed. Clarke et al. reported in 2006 that the consensus definition of a cancer stem cell that was developed for the American Association for Cancer Research (AACR) Workshop was "a cell within a tumor that possesses the capacity to self-renew and to cause the heterogeneous lineages of cancer cells that comprise the tumor." In addition, cancer stem cells could "only be defined experimentally by their ability to recapitulate the generation of a continuously growing tumor" (Clarke et al. 2006). On the other hand, MSC is also defined only by its pluripotent "ability" as described above. The next step would be the identification of the common and different features between the cells that meet the "concept" of these cells. In agreement with the theory that tumorigenesis could be seen as aberrant organogenesis (Reya et al. 2001), mutated MSCs have all that they need to source and maintain sarcoma (Honoki 2010) as recently exemplified by the effects of HMGA2 alteration in mesenchymal stem-like cells (Henriksen et al. 2010). However, it is of interest that mutated MSC or NCSC possesses malignant phenotypes, such as chemoresistance and metastasis.

The second problem is that any reliable markers for the sarcoma CSC have not been specified. Although sarcoma stem cells have been isolated and identified by various methods, no one has been able to completely eliminate non-CSCs. The final achievement in detecting CSC markers would be identifying the makers specific to sarcoma CSCs; however, only one or two markers seem too simple to represent sarcoma CSCs, which possess the multiple phenotype described above. Thus, further markers adding to current ones should be discussed to purify sarcoma CSCs more exclusively. The ideal goal would be to apply these CSC markers to practical pathological use to help make decisions regarding treatment strategies, including the current molecular therapeutics of CSCs.

For the achievement of this purpose, more evidence on clinical biology is also needed. The essence of identifying CSCs in sarcoma lies in their clinical implications. Therefore, the third problem is that there is no evidence of the correlation between sarcoma CSCs and clinical behaviors. A major goal in order to discover the best therapies against tumor progression and to address the question of CSC hypothesis would require analyzing patient samples at different stages of disease and, in particular, a follow-up of CSC numbers during and after treatment. Little evidence is available that deals exclusively with sarcoma biopsy specimens; moreover, there is still no

study in which the specimens of post-chemotherapy and metastasis have been analyzed. This is an important aspect remaining to be addressed to confirm the clinical significance of this research.

Despite the inconsistency in the definition and the CSC markers, new approaches have to be developed to overcome the most lethal characteristics, such as chemoresistance and metastasis. The final problem deals with targeting and killing these cells. Several suggestions and trials demonstrated in this chapter are summarized below.

1) Inhibition of the self-renewal pathway: The major focus has been on developmental signaling pathways, including Hedgehog, Notch, and Wnt, and novel agents inhibiting these pathways that have been used to target CSCs in multiple diseases. Clinical trials using inhibitors of the Hedgehog pathway (GDC-0449, PF-04449913, BMS-833923, IPI-926, and TAK-441), Notch pathway (RO4929097, BMS-906024, and MK0752), Wnt pathway (PRI-724 and telomerase [GRN163L]) have begun to emerge, but their efficacy against CSC function remains to be determined (Rasheed et al. 2011). Since little has been tried on sarcomas, novel trials against sarcomas would be expected. 2) Inhibition of the other molecular pathways: Although not reached to clinical trials, MEK inhibitor has been proposed for osteosarcoma CSC treatment. The evidences on the activation of MAPK pathways have emerged also in the other cancers. Then, multiple pathways including self-renewal or the other molecular pathways might be associated with the CSC phenotypes. Therefore, the most influential pathways should be discussed in each type of sarcomas. However, we have no idea how many pathways are enough to be inhibited for regulation of CSC phenotypes. Further analysis above single molecular pathways would be required. 3) Antibody drugs: Targeting antibodies specific to CSCs would be the ideal approach for the elimination of CSCs. However, a commonly used marker in CSC studies, CD133 (also known as prominin) for example, has been implied as a normal stem cell marker. Thus, targeting CD133 has the potential for significant side effects. This makes it difficult to target on the reported markers of CSCs, so that the novel markers that specify only cancer cells would be required. 4) microRNA: The use of a natural miRNA to suppress the CSC phenotypes is also a promising possibility as a new therapeutic strategy for the treatment of CSCs. miRNA also possesses the potential for use as a biomarker. The development of biomarker strategies that quantify CSCs in a serial fashion may provide novel endpoints to monitor CSC-based clinical trials. 5) Immunotherapy: The CTL-based immunotherapy targeting on SP cells has been recently proposed. Further analysis including in vivo assay or the strategy targeting on the other CSC markers would raise expectations for sarcoma treatments. 6) Others: The other alternatives include oncolytic viruses that seem to be well suited to eliminate CSCs because the viruses are

cytotoxic and not subject to the drug efflux, such as ABC transporters and defective apoptotic signaling (Cripe et al. 2009). Virotherapy could also be used to sensitize tumor cells to radiation and as tools for immunotherapy (Thorne et al. 2010). Furthermore, the stem cell niche should be analyzed and targeted because of the implications of their essential role in deciding the stem cell fate and resistance to therapeutic drugs.

CSC studies on sarcoma are in their infancy compared to studies of carcinomas and hematopoietic malignancy. In this regard, we hope for the increasing preclinical and clinical trials of targeting on sarcoma CSCs. Researchers would have to continue their efforts to provide better prognosis for many sarcoma patients all over the world.

Acknowledgments

This work was supported in part by a grant-in-aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control of Japan; a grant-in-aid for Scientific Research on Priority Areas Cancer from the Japanese Ministry of Education, Culture, Sports, Science, and Technology; and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation of Japan.

References

Adhikari, A.S., N. Agarwal, B.M. Wood, C. Porretta, B. Ruiz, R.R. Pochampally, and T. Iwakuma. 2010. CD117 and Stro-1 identify osteosarcoma tumor-initiating cells associated with metastasis and drug resistance. Cancer Res 70: 4602–4612.

Al-Hajj, M., M.S. Wicha, A. Benito-Hernandez, S.J. Morrison, and M.F. Clarke. 2003. Prospective identification of tumorigenic breast cancer cells. Proc Natl Acad Sci USA 100: 3983–3988.

Alison, M.R., S.M. Lim, and L.J. Nicholson. 2010. Cancer stem cells: problems for therapy? J Pathol 27.

Altieri, D.C. 2003. Validating survivin as a cancer therapeutic target. Nat Rev Cancer 3: 46–54.

Ambroziak, W., G. Izaguirre, and R. 1999. Pietruszko. Metabolism of retinaldehyde and other aldehydes in soluble extracts of human liver and kidney. J Biol Chem 274: 33366–33373.

Aogi, K., A. Woodman, V. Urquidi, D.C. Mangham, D.T. arin, and S. Goodison. 2000. Telomerase activity in soft-tissue and bone sarcomas. Clin Cancer Res. 6: 4776–4781.

Aoyama, T., T. Okamoto, S. Nagayama, K. Nishijo, T. Ishibe, K. Yasura, T. Nakayama, T. Nakamura, and J. Toguchida. 2004. Methylation in the core-promoter region of the chondromodulin-I gene determines the cell-specific expression by regulating the binding of transcriptional activator Sp3. J Biol Chem 279: 28789–28797.

Armstrong, L., M. Stojkovic, I. Dimmick, S. Ahmad, P. Stojkovic, N. Hole, and M. Lako. 2004. Phenotypic characterization of murine primitive hematopoietic progenitor cells isolated on basis of aldehyde dehydrogenase activity. Stem Cells 22: 1142–1151.

Artavanis-Tsakonas, S., M.D. Rand, and R.J. Lake. 1999. Notch signaling: cell fate control and signal integration in development. Science 284: 770–776.

Aryee, D.N., S. Niedan, M. Kauer, R. Schwentner, I.M. Bennani-Baiti, J. Ban, K. Muehlbacher, M. Kreppel, R.L. Walker, P. Meltzer, C. Poremba, R. Kofler, and H. Kovar. 2010. Hypoxia modulates EWS-FLI1 transcriptional signatureand enhances the malignant properties of Ewing's sarcoma cells *in vitro*, Cancer Res 70: 4015–4023.

Avilion, A.A., S.K. Nicolis, L.H. Pevny, L. Perez, N. Vivian, and R. Lovell-Badge. 2003. Multipotent cell lineages in early mouse development depend on SOX2 function. Genes Dev 17: 126–140.

Awad, O., J.T. Yustein, P. Shah, N. Gul, V. Katuri, A. O'Neill, Y. Kong, M.L. Brown, J.A. Toretsky, and D.M. Loeb. 2010. High aldh activity identifies chemotherapy-resistant Ewing's sarcoma stem cells that retain sensitivity to ews-fli1 inhibition. PLoS One 5: e13943.

Bacci, G., S. Ferrari, F. Bertoni, P. Ruggieri, P. Picci, A. Longhi, R. Casadei, N. Fabbri, C. Forni, M. Versari, and M. Campanacci. 2000. Long-term outcome for patientswith nonmetastatic osteosarcoma of the extremity treated at theIstituto Ortopedico Rizzoli according to the Istituto Ortopedico Rizzoli/osteosarcoma-2 protocol: an updated report. J Clin Oncol 18: 4016–4027.

Baksh, D., and R.S. Tuan. 2007. Canonical and non-canonical Wnts differentially affect the developmentpotential of primary isolate of human bone marrow mesenchymal stem cells. J Cell Physiol 212: 817–826.

Banas, A., T. Teratani, Y. Yamamoto, M. Tokuhara, F. Takeshita, M. Osaki, M. Kawamata, T. Kato, H. Okochi, and T. Ochiya. 2008. IFATS collection: *in vivo* therapeutic potential of human adipose tissue mesenchymal stem cells after transplantation into mice with liver injury. Stem Cells 26: 2705–2712.

Barker, N., M. Huch, P. Kujala, M. van de Wetering, H.J. Snippert, J.H. van Es, T. Sato, D.E. Stange, H. Begthel, M. van den Born, E. Danenberg, S. van den Brink, J. Korving, A. Abo, P.J. Peters, N. Wright, R. Poulsom, and H. Clevers. 2010. Lgr5(+ve) stem cells drive self-renewal in the stomach and build long-lived gastric units *in vitro*. Cell Stem Cell 6: 25–36.

Bartel, D.P. 2004. MicroRNAs: genomics, biogenesis, mechanism, and function. Cell 116: 281–297.

Basu-Roy, U., E. Seo, L. Ramanathapuram, T.B. Rapp, J.A. Perry, S.H. Orkin, A. Mansukhani, and C. Basilico. 2011. Sox2 maintains self renewal of tumor-initiating cells in osteosarcomas. Oncogene. doi: 10.1038/onc.2011.405

Bennani-Baiti, I.M., D.N. Aryee, J. Ban, I. Machado, M. Kauer, K. Mühlbacher, G. Amann, A. Llombart-Bosch, and H. Kovar. 2011. Notch signalling is off and is uncoupled from HES1 expression in Ewing's sarcoma. J Pathol 225: 353–363.

Bielack, S., B. Kempf-Bielack, D. Schwenzer, T. Birkfellner, G. Delling, V. Ewerbeck, G.U. Exner, N. Fuchs, U. Göbel, N. Graf, U. Heise, K. Helmke, A.R. von Hochstetter, H. Jürgens, R. Maas, N. Münchow, M. Salzer-Kuntschik, J. Treuner, U. Veltmann, M. Werner, W. Winkelmann, A. Zoubek, and R. Kotz. 1999. Neoadjuvanttherapy for localized osteosarcoma of extremities: results from the Cooperative osteosarcoma study group COSS of 925 patients. Klin Padiatr 211: 260–270.

Bolós, V., J. Grego-Bessa, and J.L. de la Pompa. 2007. Notch signaling in development and cancer. Endocr Rev 28: 339–363.

Brady, M.S., G. Perino, G. Tallini, P. Russo, and J.M. Woodruff. 1996. Malignant mesenchymoma. Cancer 77: 467–473.

Carpentino, J.E., M.J. Hynes, H.D. Appelman, T. Zheng, D.A. Steindler, E.W. Scott, and E.H. Huang. 2009. Aldehyde dehydrogenase-expressing colon stem cells contribute to tumorigenesis in the transition from colitis to cancer. Cancer Res 69: 8208–8215.

Catton, C., B. O'Sullivan, R. Bell, N. Laperriere, B. Cummings, V. Fornasier, and J. Wunder. 1996. Chordoma: Long-term follow-up after radical photonirradiation. Radiother Oncol 41: 67–72.

Cha, E.J. 2011. Dedifferentiated liposarcoma of the small bowel mesentery presenting as a submucosal mass. World J Gastrointest Oncol 3: 116–118.

Chambers, P.W., and C.P. Schwinn. 1979. Chordoma. A clinicopathologic study of metastasis. Am J Clin Pathol 72: 765–776.

Charafe-Jauffret, E., C. Ginestier, F. Iovino, C. Tarpin, M. Diebel, B. Esterni, G. Houvenaeghel, J.M. Extra, F. Bertucci, J. Jacquemier, L. Xerri, G. Dontu, G. Stassi, Y. Xiao, S.H. Barsky, D. Birnbaum, P. Viens, and M.S. Wicha. 2010. Aldehyde dehydrogenase 1-positive cancer stem cells mediate metastasis and poor clinical outcome in inflammatory breast cancer. Clin Cancer Res. 16: 45–55.

Chiba, T., S. Miyagi, A. Saraya, R. Aoki, A. Seki, Y. Morita, Y. Yonemitsu, O. Yokosuka, H. Taniguchi, H. Nakauchi, and A. Iwama. 2008. The polycomb gene product bmi1 contributes to the maintenance of tumor-initiating side population cells in hepatocellular carcinoma. Cancer Res 68: 7742–7749.

Cheng, J.X., B.L. Liu, and X. Zhang. 2009. How powerful is CD133 as a cancer stem cell marker in brain tumors? Cancer Treat Rev 35: 403–408.

Chiyomaru, T., H. Enokida, S. Tatarano, K. Kawahara, Y. Uchida, K. Nishiyama, L. Fujimura, N. Kikkawa, N. Seki, and M. Nakagawa. 2010. miR-145 and miR-133a function as tumor suppressors and directly regulate FSCN1 expression in bladder cancer. Br J Cancer 102: 883–891.

Chugh, R., H. Tawbi, D.R. Lucas, J.S. Biermann, S.M. Schuetze, and L.H. Baker. 2007. Chordoma: the nonsarcoma primary bone tumor. Oncologist 12: 1344–1350.

Clarke, M.F., J.E. Dick, P.B. Dirks, C.J. Eaves, C.H. Jamieson, D.L. Jones, J. Visvader, I.L. Weissman, and G.M. Wahl. 2006. Cancer stem cells—perspectives on current status and future directions: AACR Workshop on cancer stem cells. Cancer Res 66: 9339–9344.

Clevers, H. 2011. The cancer stem cell: premises, promises and challenges. Nat Med 17: 313–319.

Collins, A.T., P.A. Berry, C. Hyde, M.J. Stower, and N.J. Maitland. 2005. Prospective identification of tumorigenic prostate cancer stem cells. Cancer Res 65: 10946–10951.

Cripe, T.P., P.Y. Wang, P. Marcato, Y.Y. Mahller, and P.W. Lee. 2009. Targeting cancer-initiating cells with oncolytic viruses. Mol Ther 17: 1677–1682.

Croce, C.M. 2009. Causes and consequences of microRNA dysregulation in cancer. Nat Rev Genet 10: 704–714.

Dahlin, D.C., and J.W. Beabout. 1971. Dedifferentiation of lowgrade chondrosarcomas. Cancer 28: 461–466.

Da Silva Meirelles, L., T.T. Sand, R.J. Harman, D.P. Lennon, and A.I. Caplan. 2009. MSC frequency correlates with blood vessel density in equine adipose tissue. Tissue Eng Part A 15: 221–229.

David, E., F. Blanchard, M.F. Heymann, G. De Pinieux, F. Gouin, F. Rédini, and D. Heymann. 2011. The Bone Niche of Chondrosarcoma: A Sanctuary for Drug Resistance, Tumor Growth and also a Source of New Therapeutic Targets. Sarcoma 2011: 932451.

De Giovanni, C., L. Landuzzi, G. Nicoletti, P.L. Lollini, and P. Nanni. 2009. Molecularand cellular biology of rhabdomyosarcoma. Future Oncol 5: 1449–1475.

De Jong, J., and L.H. Looijenga. 2006. Stem cell marker OCT3/4 in tumor biology and germ cell tumor diagnostics: history and future. Crit Rev Oncog 12: 171–203.

Di Fiore, R., A. Santulli, R.D. Ferrante, M. Giuliano, A. De Blasio, C. Messina, G. Pirozzi, V. Tirino, G. Tesoriere, and R. Vento. 2009. Identification and expansion of human osteosarcoma-cancer-stem cells by long-term 3-aminobenzamide treatment. J Cell Physiol 219: 301–313.

Ding, Y., E.A. Boguslawski, B.D. Berghuis, J.J. Young, Z. Zhang, K. Hardy, K. Furge, E. Kort, A.E. Frankel, R.V. Hay, J.H. Resau, and N.S. Duesbery. 2008. Mitogen-activated protein kinase kinase signaling promotes growth and vascularization of fibrosarcoma. Mol Cancer Ther 7: 648–658.

Dorfman, H.D., and B. Czerniak. 1995. Bone cancers. Cancer 75: 203–210.

Downing, J.R., A. Khandekar, S.A. Shurtleff, D.R. Head, D.M. Parham, B.L. Webber, A.S. Pappo, M.G. Hulshof, W.P. Conn, and D.N. Shapiro. 1995. Multiplex RT-PCR assay

for the differential diagnosis of alveolar rhabdomyosarcoma and Ewing's sarcoma. Am J Pathol 146: 626–634.

Duester, G., F.A. Mic, and A. Molotkov. 2003. Cytosolic retinoid dehydrogenases govern ubiquitous metabolism of retinol to retinaldehyde followed by tissue-specific metabolism to retinoic acid. Chem Biol Interact 143–144: 201–210.

Eilber, F.C., M.F. Brennan, F.R. Eilber, J.J Eckardt, S.R. Grobmyer, E. Riedel, C. Forscher, R.G. Maki, and S. Singer. 2007. Chemotherapy is associated with improved survival in adult patients with primary extremity synovial sarcoma. Ann Surg 246: 105–113.

Engin, F., Z. Yao, T. Yang, G. Zhou, T. Bertin, M.M. Jiang, Y. Chen, L. Wang, H. Zheng, R.E. Sutton, B.F. Boyce, and B. Lee. 2008. Dimorphic effects of Notch signaling in bone homeostasis. Nat Med 14: 299–305.

Entz-Werle, N., L. Marcellin, M.P. Gaub, E. Guerin, A. Schneider, P. Berard-Marec, C. Kalifa, L. Brugiere, H. Pacquement, C. Schmitt, M.D. Tabone, C. Jeanne-Pasquier, P. Terrier, F. Dijoud, P. Oudet, P. Lutz, and A. Babin-Boilletot. 2005. Prognostic significance of allelic imbalance at the c-kit gene locus and c-kit overexpression by immunohistochemistry in pediatric osteosarcomas. J Clin Oncol 23: 2248–2255.

Entz-Werle, N., M.P. Gaub, T. Lavaux, L. Marcellin, N. Metzger, P. Marec-Berard, C. Schmitt, L. Brugiere, C. Kalifa, M.D. Tabone, H. Pacquement, P. Gentet, P. Lutz, P. Oudet, and A. Babin. 2007. KIT gene in pediatric osteosarcomas: could it be a new therapeutic target? Int J Cancer 120: 2510–2516.

Esiashvili, N., M. Goodman, and R.B. Marcus Jr. 2008. Changes in incidence and survival of Ewing sarcoma patients over the past 3 decades: Surveillance Epidemiology and End Results data. J Pediatr Hematol Oncol 30: 425–430.

Esnaola, N.F., B.P. Rubin, E.H. Baldini, N. Vasudevan, G.D. Demetri, C.D. Fletcher, and S. Singer. 2001. Response to chemotherapy and predictors of survival in adult rhabdomyosarcoma. Ann Surg 234: 215–223.

Evans, H.L., A.G. Ayala, and M.M. Romsdahl. 1977. Prognostic factors in chondrosarcoma of bone: a clinicopathologic analysis with emphasis on histologic grading. Cancer 40: 818–831.

Evans, H.L. 1979. Liposarcoma: a study of 55 cases with areassessment of its classification. Am J Surg Pathol 3: 507–523.

Farshid, G., M. Pradhan, J. Goldblum, and S.W. Weiss. 2002. Leiomyosarcoma of somatic soft tissues: a tumor of vascular origin with multivariate analysis of outcome in 42 cases. Am J Surg Pathol 26: 14–24.

Ferguson, W., and A. Goorin. 2001. Current treatment of osteosarcoma. Cancer Invest 19: 292–315.

Ferraresi, V., C. Nuzzo, C. Zoccali, F. Marandino, A. Vidiri, N. Salducca, M. Zeuli, D. Giannarelli, F. Cognetti, and R. Biagini. 2010. Chordoma: clinical characteristics, management and prognosis of a case series of 25 patients. BMC Cancer 10: 22.

Ferrari, A., G. Bisogno, R. Alaggio, G. Cecchetto, P. Collini, A. Rosolen, C. Meazza, P. Indolfi, A. Garaventa, L. De Sio, P. D'Angelo, P. Tamaro, M. Casanova, and M. Carli. 2008. Synovial sarcoma of children and adolescents: the prognostic role of axial sites. Eur J Cancer 44: 1202–1209.

Ferrari, S., E. Palmerini, E.L. Staals, M. Mercuri, B. Franco, P. Picci, and G. Bacci. 2009. The treatment of nonmetastatic high grade osteosarcoma of the extremity: review of the Italian Rizzoli experience. Impact on the future. Cancer Treat Res 152: 275–287.

Fibbe, W.E. 2002. Mesenchymal stem cells. A potential source for skeletal repair. Ann Rheum Dis 61: ii29–31.

Fletcher, C.D.M., K.K. Unni, and F. Mertens. 2002. World Health Organisation classification of tumors. Pathology and genetics of tumors of soft tissue and bone. IARC Press, Lyon, France.

Fleming, G.F., P.S. Heimann, J.K. Stephens, M.A. Simon, M.K. Ferguson, R.S. Benjamin, and B.L. Samuels. 1993. Dedifferentiated chordoma. Response toaggressive chemotherapy in two cases. Cancer 72: 714–718.

Friedenstein, A.J., J.F. Gorskaja, and N.N. Kulagina. 1976. Fibroblast precursors in normal and irradiated mouse hematopoietic organs. ExpHematol 4: 267–274.

Fuchs, D., V. Daniel, M. Sadeghi, G. Opelz, and C. Naujokat. 2010. Salinomycin overcomes ABC transporter-mediated multidrug and apoptosis resistance in human leukemia stem cell-like KG-1a cells. Biochem Biophys Res Commun 394: 1098–1104.

Fujii, H., K. Honoki, T. Tsujiuchi, A. Kido, K. Yoshitani, and Y. Takakura. 2009. Sphere-forming stem-like cell populations with drug resistance in human sarcoma cell lines. Int J Oncol 34: 1381–1386.

Furlong, M.A., T. Mentzel, and J.C. Fanburg-Smith. 2001. Pleomorphic rhabdomyosarcoma in adults: a clinicopathologic study of 38 cases with emphasis on morphologic variants and recent skeletal muscle-specific markers. Mod Pathol 14: 595–603.

Galli, R., U. Borello, A. Gritti, M.G. Minasi, C. Bjornson, M. Coletta, M. Mora, M.G. De Angelis, R. Fiocco, G. Cossu, and A.L. Vescovi. 2000. Skeletal myogenic potential of human and mouse neural stem cells. Nat Neurosci 3: 986–991.

Geller, D.S., and R. Gorlick. 2010. Osteosarcoma: a review of diagnosis, management, and treatment strategies. Clin Adv Hematol Oncol 8: 705–718.

Gelderblom, H., P.C. Hogendoorn, S.D. Dijkstra, C.S. van Rijswijk, A.D. Krol, A.H. Taminiau, and J.V. Bovée. 2008. The clinical approach towards chondrosarcoma. Oncologist 13: 320–329.

Gibbs, C.P., V.G. Kukekov, J.D. Reith, O. Tchigrinova, O.N. Suslov, E.W. Scott, S.C. Ghivizzani, T.N. Ignatova, and D.A. Steindler. 2005. Stem-like cells in bone sarcomas: implications for tumorigenesis. Neoplasia 7: 967–976.

Gidekel, S., G. Pizov, Y. Bergman, and E. Pikarsky. 2003. Oct-3/4 is a dose-dependent oncogenic fate determinant. Cancer Cell 4: 361–370.

Goodell, M.A., M. Rosenzweig, H. Kim, D.F. Marks, M. DeMaria, G. Paradis, S.A. Grupp, C.A. Sieff, R.C. Mulligan, and R.P. Johnson. 1997. Dye efflux studies suggest that hematopoietic stem cells expressing low or undetectable levels of CD34 antigen exist in multiple species. Nat Med 3: 1337–1345.

Gregory, C.A., H. Singh, A.S. Perry, and D.J. Prockop. 2003. The Wnt signaling inhibitor dickkopf-1 is required for reentry into the cell cycle of human adult stem cells from bone marrow. J Biol Chem 278: 28067–28078.

Gronthos, S., S.E. Graves, S. Ohta, and P.J. Simmons. 1994. The STRO-1+ fractionof adult human bone marrow contains the osteogenic precursors. Blood 84: 4164–4173.

Guo, L., R.C. Zhao, and Y. Wu. 2011. The role of microRNAs in self-renewal and differentiation of mesenchymal stem cells. Exp Hematol 39: 608–616.

Gupta, P.B., T.T. Onder, G. Jiang, K. Tao, C. Kuperwasser, R.A. Weinberg, and E.S. Lander. 2009. Identification of selective inhibitors ofcancer stem cells by high-throughput screening. Cell 138: 645–659.

Halldorsson, A., S. Brooks, S. Montgomery, and S. Graham. 2009. Lung metastasis 21 years after initial diagnosis of osteosarcoma: a case report. J Med Case Reports 3: 9298.

Heddleston, J.M., Z. Li, J.D. Lathia, S. Bao, A.B. Hjelmeland, and J.N. Rich. 2010. Hypoxia inducible factors in cancer stem cells. Br J Cancer 102: 789–795.

Henriksen, J., M. Stabell, L.A. Meza-Zepeda, S.A. Lauvrak, M. Kassem, and O. Myklebost. 2010. Identification of targetgenes for wild type and truncated HMGA2 in mesenchymal stem-likecells. BMC Cancer 10: 329.

Hermann, P.C., S.L. Huber, T. Herrler, A. Aicher, J.W. Ellwart, M. Guba, C.J. Bruns, and C. Heeschen. 2007. Distinct populations of cancer stem cells determine tumor growth and metastatic activity in human pancreatic cancer. Cell Stem Cell 1: 313–323

Hess, D.A., T.E. Meyerrose, L. Wirthlin, T.P. Craft, P.E. Herrbrich, M.H. Creer, and J.A. Nolta. 2004. Functional characterization of highly purified human hematopoietic repopulating cells isolated according to aldehyde dehydrogenase activity. Blood 104: 1648–1655.

Higinbotham, N.L., R.F. Phillips, H.W. Farr, and H.O. Hustu. 1967. Chordoma. Thirty-five year study at Memorial Hospital. Cancer 20: 1841–1850.

Hirotsu, M., T. Setoguchi, Y. Matsunoshita, H. Sasaki, H. Nagao, et al. 2009. Tumor formation by single fibroblast growth factor receptor 3-positiverhabdomyosarcoma-initiating cells. Br J Cancer 101: 2030–2037.

Honoki, K., H. Fujii, A. Kubo, A. Kido, T. Mori, Y. Tanaka, and T. Tsujiuchi. 2010. Possible involvement of stem-like populations with elevated ALDH1 in sarcomas for chemotherapeutic drug resistance. Oncol Rep 24: 501–505.

Honoki, K. 2010. Do stem-like cells play a role in drug resistance of sarcomas? Expert Rev Anticancer Ther 10: 261–270.

Huang, X., and J. Saint-Jeannet. 2004. Induction of the neural crest and the opportunities of life on the edge. Developmental Biology 275: 1–11.

Huber, M.A., N. Kraut, and H. Beug. 2005. Molecular requirements for epithelial-mesenchymal transition during tumor progression. Curr Opin Cell Biol 17: 548–558.

Ibrahim, S.F., A.H. Diercks, T.W. Petersen, and G. van den Engh. 2007. Kinetic analyses as a critical parameter in defining the side population (SP) phenotype. Exp Cell Res 313: 1921–1926.

Ischenko, I., H. Seeliger, M. Schaffer, K.W. Jauch, and C.J. Bruns. 2008. Cancer stem cells: how can we target them? Curr Med Chem 15: 3171–3184.

Iwasaki, H., and T. Suda. 2009. Cancer stem cells and their niche. Cancer Sci 100: 1166–1172.

James, G., M. Crocker, A. King, I. Bodi, A. Ibrahim, and B.P. Chitnavis. 2008. Malignant triton tumors of the spine. J Neurosurg Spine 8: 567–573.

Jamil, N., S. Howie, and D.M. Salter. 2010. Therapeutic molecular targets in human chondrosarcoma. Int J Exp Pathol 91: 387–393.

Jedlicka, P. 2010. Ewing Sarcoma, an enigmatic malignancy of likely progenitor cell origin, driven by transcription factor oncogenic fusions. Int J Clin Exp Pathol 19 3: 338–347.

Jones, R.L., C. Fisher, O. Al-Muderis, and I.R. Judson. 2005. Differential sensitivity of liposarcoma subtypes to chemotherapy. Eur J Cancer 41: 2853–2860.

Jiang, X., Y. Gwye, D. Russell, C. Cao, D. Douglas, L. Hung, H. Kovar, T.J. Triche, E.R. Lawlor. 2010. CD133 expression in chemo-resistant Ewing sarcoma cells. BMC Cancer 26 10: 116.

Katoh, M., C. Shaw, Q. Xu, N. Van Driessche, T. Morio, H. Kuwayama, S. Obara, H. Urushihara, Y. Tanaka, and G. Shaulsky. 2004. An orderly retreat: Dedifferentiation is a regulated process. Proc Natl Acad Sci USA 101: 7005–7010.

Kawamoto, E.H., N. Weidner, R.M. Agostini Jr, and R. Jaffe. 1987. Malignant ectomesenchymoma of soft tissue report of two cases and review of the literature. Cancer 59: 1791–1802.

Kawai, A., J. Woodruff, J.H. Healey, M.F. Brennan, C.R. Antonescu, and M. Ladanyi. 1998. SYT-SSX gene fusion as a determinant of morphology and prognosis in synovial sarcoma. N Engl J Med 338: 153–160.

Kelly, S.E., A. Di Benedetto, A. Greco, C.M. Howard, V.E. Sollars, D.A. Primerano, J.V. Valluri, and P.P. Claudio. 2010. Rapid selection and proliferation of CD133+ cells from cancer cell lines: chemotherapeutic implications. PLoS One 5: e10035.

Kim, V.N., J. Han, and M.C. Siomi. 2009. Biogenesis of small RNAs in animals. Nat Rev Mol Cell Biol 10: 126–139.

Kosaka, N., H. Iguchi, and T. Ochiya. 2010. Circulating microRNA in body fluid: a new potential biomarker for cancer diagnosis and prognosis. Cancer Sci 101: 2087–2092.

Kubo, T., T. Sugita, S. Shimose, T. Matsuo, K. Arihiro, and M. Ochi. 2008. Expression of hypoxia-inducible factor-1alpha and its relationship to tumor angiogenesis and cell proliferation in cartilage tumors. J Bone Joint Surg Br 90: 364–370.

Kuhn, N.Z., and R.S. Tuan. 2010. Regulation of stemness and stem cell niche ofmesenchymal stem cells: implications in tumorigenesis and metastasis. J Cell Physiol 222: 268–277.

Lapidot, T., C. Sirard, J. Vormoor, B. Murdoch, T. Hoang, J. Caceres-Cortes, M. Minden, B. Paterson, M.A. Caligiuri, and J.E. Dick. 1994. A cell initiating human acute myeloid leukemia after transplantation into SCID mice. Nature 367: 645–648.

Le Douarin, N.M., G.W. Calloni, and E. Dupin. 2008. The stem cells of the neural crest. Cell Cycle 7: 1013–1019.

Lee, A., J.D. Kessler, T.A. Read, C. Kaiser, D. Corbeil, W.B. Huttner, J.E. Johnson, and R.J. Wechsler-Reya. 2005. Isolation of neural stem cells from the postnatal cerebellum. Nat Neurosci 8: 723–729.

Lehnhardt, M., A. Daigeler, H.H. Homann, V. Schwaiberger, O. Goertz, C. Kuhnen, H.U. Steinau. 2009. MFH revisited: outcome after surgical treatment of undifferentiated pleomorphic or not otherwise specified (NOS) sarcomas of the extremities—an analysis of 140 patients. Langenbecks Arch Surg 394: 313–320.

Levings, P.P., S.V. McGarry, T.P. Currie, D.M. Nickerson, S. McClellan, S.C. Ghivizzani, D.A. Steindler, and C.P. Gibbs. 2009. Expression of an exogenous human Oct-4 promoter identifies tumor-initiating cells in osteosarcoma. Cancer Res 69: 5648–5655.

Li, C., D.G. Heidt, P. Dalerba, C.F. Burant, L. Zhang, V. Adsay, M. Wicha, M.F. Clarke, and D.M. Simeone. 2007. Identification of pancreatic cancer stem cells. Cancer Res 67: 1030–1037.

Li, J., W. Liu, K. Zhao, Y. Zhang, X. Li, Q. Yang, Z. Li, and J. Li. 2009. Diallyl trisulfide reverses drug resistance and lowers the ratio of CD133+ cells in conjunction with methotrexate in a human osteosarcoma drug-resistant cell subline. Mol Med Report 2: 245–252.

Lin, G.L., and K.D. Hankenson. 2011. Integration of BMP, Wnt, and notch signaling pathways in osteoblast differentiation. J Cell Biochem 112: 3491–3501.

Lindner, N.J., O. Ramm, A. Hillmann, R. Roedl, G. Gosheger, C. Brinkschmidt, H. Juergens, and W. Winkelmann. 1999. Limb salvage and outcome of osteosarcoma. The University of Muenster experience. Clin Orthop Relat Res 358: 83–89.

Liu, X., Y. Sun, J. Guo, H. Ma, J. Li, B. Dong, G. Jin, J. Zhang, J. Wu, L. Meng, and C. Shou. 2006. Expression of hiwi gene in human gastric cancer wasassociated with proliferation of cancer cells. Int J Cancer 118: 1922–1929.

Longhi, A., C. Errani, M. De Paolis, M. Mercuri, and G. Bacci. 2006. Primary boneosteosarcoma in the pediatric age: state of the art. Cancer Treat Rev 32: 423–436.

Maeda, S., H. Shinchi, H. Kurahara, Y. Mataki, K. Maemura, M. Sato, S. Natsugoe, T. Aikou, and S. Takao. 2008. CD133 expression is correlated with lymph node metastasis and vascular endothelial growth factor-c expression in pancreatic cancer. Br J Cancer 98: 1389–1397.

Malanchi, I., H. Peinado, D. Kassen, T. Hussenet, D. Metzger, P. Chambon, M. Huber, D. Hohl, A. Cano, W. Birchmeier, and J. Huelsken. 2008. Cutaneous cancer stem cell maintenance is dependent on beta-catenin signalling. Nature 452: 650–653.

Mankin, H.J., J. Casas-Ganem, J.I. Kim, M.C. Gebhardt, F.J. Hornicek, and E.N. Zeegen. 2004. Leiomyosarcoma of somatic soft tissues. Clin Orthop Relat Res 421: 225–231.

Marina, N., M. Gebhardt, L. Teot, and R. Gorlick. 2004. Biology and therapeutic advances for pediatric osteosarcoma. Oncologist 9: 422–441.

Masui, S., Y. Nakatake, Y. Toyooka, D. Shimosato, R. Yagi, K. Takahashi, H. Okochi, A. Okuda, R. Matoba, A.A. Sharov, M.S. Ko, and H. Niwa. 2007. Pluripotency governed by Sox2 via regulation of Oct3/4 expression in mouse embryonic stem cells. Nat Cell Biol 9: 625–635.

Matsui, W., C.A. Huff, Q. Wang, M.T. Malehorn, J. Barber, Y. Tanhehco, B.D. Smith, C.I. Civin, and R.J. Jones. 2004. Characterization of clonogenic multiple myeloma cells. Blood 103: 2332–2336.

Matushansky, I., R.G. Maki, and C. Cordon-Cardo. 2008. A context dependent role for Wnt signaling in tumorigenesis and stem cells. Cell Cycle 7: 720–724.

Matushansky, I., E. Hernando, N.D. Socci, J.E. Mills, T.A. Matos, M.A. Edgar, S. Singer, R.G. Maki, and C. Cordon-Cardo. 2007. Derivation of sarcomas from mesenchymal stem cells via inactivation of the Wnt pathway. J Clin Invest 117: 3248–3257.

McDowell, H.P. 2003. Update on childhood rhabdomyosarcoma. Arch Dis Child 88: 354–357.

Merlino, G., and C. Khanna. 2007. Fishing for the origins of cancer. Genes Dev 21: 1275–1279.

Meyers, P.A., R. Gorlick, G. Heller, E. Casper, J. Lane, A.G. Huvos, and J.H. Healey. 1998. Intensification of preoperativechemotherapy for osteogenic sarcoma: results of the Memorial Sloan-Kettering (T12) protocol. J Clin Oncol 16: 2452–2458.

Meyers, P.A., C.L. Schwartz, M.D. Krailo, J.H. Healey, M.L. Bernstein, D. Betcher, W.S. Ferguson, M.C. Gebhardt, A.M. Goorin, M. Harris, E. Kleinerman, M.P. Link, H. Nadel, M. Nieder, G.P. Siegal, M.A. Weiner, R.J. Wells, R.B. Womer, and H.E. Grier. 2008. Children's Oncology Group. Osteosarcoma: the addition of muramyl tripeptide to chemotherapy improves overall survival—a report from the Children's Oncology Group. J Clin Oncol 26: 633–638.

Misra, A., N. Mistry, R. Grimer, and F. Peart. 2009. The management of soft tissue sarcoma. J Plast Reconstr Aesthet Surg 62: 161–174.

Mizrak, D., M. Brittan, and M.R. Alison. 2008. CD133: Molecule of the moment. J Pathol 214: 3–9.

Mohseny, A.B., K. Szuhai, S. Romeo, E.P. Buddingh, I. Briaire-de Bruijn, D. de Jong, M. van Pel, A.M. Cleton-Jansen, and P.C. Hogendoorn. 2009. Osteosarcoma originates from mesenchymal stem cells in consequence of aneuploidization and genomic loss of Cdkn2. J Pathol 219: 294–305.

Monk, M., and C. Holding. 2001. Human embryonic genes re-expressed in cancer cells. Oncogene 20: 8085–8091.

Morrison, S.J., P.M. White, C. Zock, and D.J. Anderson. 1999. Prospective identification, isolation by flow cytometry, and *in vivo* self-renewal of multipotent mammalian neural crest stem cells. Cell 96: 737–749.

Murase, M., M. Kano, T. Tsukahara, A. Takahashi, T. Torigoe, S. Kawaguchi, S. Kimura, T. Wada, Y. Uchihashi, T. Kondo, T. Yamashita, and N. Sato. 2009. Side population cells have the characteristics of cancer stem-like cells/cancer-initiating cells in bone sarcomas. Br J Cancer 101: 1425–1432.

Nagao, H., K. Ijiri, M. Hirotsu, Y. Ishidou, T. Yamamoto, S. Nagano, T. Takizawa, K. Nakashima, S. Komiya, T. Setoguchi. 2011. Role of GLI2 in the growth of human osteosarcoma. J Pathol 224: 169–179.

Nagoshi, N., S. Shibata, M. Nakamura, Y. Matsuzaki, Y. Toyama, and H. Okano. 2009. Neural crest-derived stem cells display a wide variety of characteristics. J Cell Biochem 107: 1046–1052.

Nagoshi, N., S. Shibata, Y. Kubota, M. Nakamura, Y. Nagai, E. Satoh, S. Morikawa, Y. Okada, Y. Mabuchi, H. Katoh, S. Okada, K. Fukuda, T. Suda, Y. Matsuzaki, Y. Toyama, and H. Okano. 2008. Ontogeny and multipotency of neural crest-derived stem cells in mouse bone marrow, dorsal root ganglia, and whisker pad. Cell Stem Cell 2: 392–403.

Naka, N., S. Takenaka, N. Araki, T. Miwa, N. Hashimoto, K. Yoshioka, S. Joyama, K. Hamada, Y. Tsukamoto, Y. Tomita, T. Ueda, H. Yoshikawa, and K. Itoh. 2010. Synovial sarcoma is a stem cell malignancy. Stem Cells 28: 1119–1131.

Ng, T.L., A.M. Gown, T.S. Barry, M.C. Cheang, A.K. Chan, D.A. Turbin, F.D. Hsu, R.B. West, and T.O. Nielsen. 2005. Nuclear beta-catenin in mesenchymal tumors. Mod Pathol 18: 68–74.

Osaki, M., F. Takeshita, Y. Sugimoto, N. Kosaka, Y. Yamamoto, Y. Yoshioka, E. Kobayashi, T. Yamada, A. Kawai, T. Inoue, H. Ito, M. Oshimura, and T. Ochiya. 2011. MicroRNA-143 regulates human osteosarcoma metastasis by regulating matrix metalloprotease-13 expression. Mol Ther 19: 1123–1130.

Ozaki, T., A. Hillmann, N. Lindner, S. Blasius, and W. Winkelmann. 1997. Chondrosarcoma of the pelvis. Clin Orthop Relat Res 337: 226–239.

Pardal, R., M.F. Clarke, and S.J. Morrison. 2003. Applying the principles of stem-cell biology to cancer. Nat Rev Cancer 3: 895–902.

Patrawala, L., T. Calhoun, R. Schneider-Broussard, J. Zhou, K. Claypool, and D.G. Tang. 2005. Side population is enriched in tumorigenic, stem-like cancer cells, whereas ABCG2+ and ABCG2- cancer cells are similarly tumorigenic. Cancer Res 65: 6207–6219.

Pearce, D.J., D. Taussig, C. Simpson, K. Allen, A.Z. Rohatiner, T.A. Lister, and D. Bonnet. 2005. Characterization of cells with a high aldehyde dehydrogenase activity from cord blood and acute myeloid leukemia samples. Stem Cells 23: 752–760.

Pendino, F., I. Tarkanyi, C. Dudognon, J. Hillion, M. Lanotte, J. Aradi, and E. Ségal-Bendirdjian. 2006. Telomeres and telomerase: Pharmacological targets for new anticancer strategies? Curr Cancer Drug Targets 6: 147–180.

Pevny, L.H., and S.K. Nicolis. 2010. Sox2 roles in neural stem cells. Int J Biochem Cell Biol 42: 421–424.

Plaisant, M., S. Giorgetti-Peraldi, M. Gabrielson, A. Loubat, C. Dani, and P. Peraldi. Inhibition of hedgehog signaling decreases proliferation and clonogenicity of human mesenchymal stem cells. PLoS One 6: e16798.

Provisor, A., L. Ettinger, J. Nachman, M.D. Krailo, J.T. Makley, and E.J. Yunis. 1997. Treatment of nonmetastatic osteosarcoma of the extremity with preoperative andpostoperative chemotherapy: a report from the Children's Cancer Group. J Clin Oncol 15: 76–84.

Qiao, D., A.M. Zeeman, W. Deng, L.H. Looijenga, and H. Lin. 2002. Molecular characterization of hiwi, a human member of thepiwi gene family whose overexpression is correlated toseminomas. Oncogene 21: 3988–3999.

Que, J., X. Luo, R.J. Schwartz, and B.L. Hogan. 2009. Multiple roles for Sox2 in the developing and adult mouse trachea. Development 136: 1899–1907.

Raimondi, L., R. Ciarapica, M. De Salvo, F. Verginelli, M. Gueguen, C. Martini, L. De Sio, G. Cortese, M. Locatelli, T.P. Dang, N. Carlesso, L. Miele, S. Stifani, I. Limon, F. Locatelli, and R. Rota. 2011. Inhibition of Notch3 signalling induces rhabdomyosarcoma cell differentiation promoting p38 phosphorylation and p21(Cip1) expression and hampers tumor cell growth *in vitro* and *in vivo*. Cell Death Differ 25.

Rasheed, Z.A., J. Yang, Q. Wang, J. Kowalski, I. Freed, C. Murter, S.M. Hong, J.B. Koorstra, N.V. Rajeshkumar, X. He, M. Goggins, C. Iacobuzio-Donahue, D.M. Berman, D. Laheru, A. Jimeno, M. Hidalgo, A. Maitra, and W. Matsui. 2010. Prognostic significance of tumorigenic cells with mesenchymal features in pancreatic adenocarcinoma. J Natl Cancer Inst 102: 340–351.

Rasheed, Z.A., J. Kowalski, B.D. Smith, and W. Matsui. 2011. Concise review: Emerging concepts in clinical targeting of cancer stem cells. Stem Cells 29: 883–887.

Reya, T., S.J. Morrison, M.F. Clarke, and I.L. Weissman. 2001. Stem cells, cancer, and cancerstem cells. Nature 414: 105–111.

Reynolds, B.A., and S. Weiss. 1992. Generation of neurons and astrocytes from isolated cells of the adult mammalian central nervous system. Science 255: 1707–1710.

Ricci-Vitiani, L., D.G. Lombardi, E. Pilozzi, M. Biffoni, M. Todaro, C. Peschle, and R. De Maria. 2007. Identification and expansion of human colon-cancer-initiating cells. Nature 445: 111–115.

Riggi, N., M.L. Suvà, C. De Vito, P. Provero, J.C. Stehle, K. Baumer, L. Cironi, M. Janiszewska, T. Petricevic, D. Suvà, S. Tercier, J.M. Joseph, L. Guillou, and I. Stamenkovic. EWS-FLI-1 modulates miRNA145 and SOX2 expression to initiate mesenchymal stem cell reprogrammingtoward Ewing sarcoma cancer stem cells. Genes Dev 24: 916–932.

Rot, S., H. Taubert, M. Bache, T. Greither, P. Wurl, A.W. Eckert, J. Schubert, D. Vordermark, and M. Kappler M. 2011. A novel splice variant of the stem cell marker LGR5/GPR49 is correlated with the risk of tumor-related death in soft-tissue sarcoma patients. BMC Cancer 11: 429.

Sadikovic, B., C. Graham, M. Ho, M. Zielenska, and G.R. Somers. 2011. Immunohistochemical expression and cluster analysis of mesenchymal and neural stem cell-associated proteins in pediatric soft tissue sarcomas. Pediatr Dev Pathol. 14: 259–272.

Sasaki, K., T. Hitora, O. Nakamura, R. Kono, and T. Yamamoto. 2011. The role of MAPK pathway in bone and soft tissue tumors. Anticancer Res 31: 549–553.

Scott, S.M., H.M. Reiman, D.J. Pritchard, and D.M. Ilstrup. 1989. Soft tissue fibrosarcoma. A clinicopathologic study of 132 cases. Cancer 64: 925–931.

Shi, S., and S. Gronthos. 2003. Perivascular niche of postnatal mesenchymal stem cells in human bone marrow and dental pulp. J Bone Miner Res 18: 696–704.

Shima, Y., T. Okamoto, T. Aoyama, K. Yasura, T. Ishibe, K. Nishijo, K.R. Shibata, Y. Kohno, K. Fukiage, S. Otsuka, D. Uejima, T. Nakayama, T. Nakamura, T. Kiyono, and J. Toguchida. 2007. *In vitro* transformation of mesenchymal stem cells by oncogenic H-rasVal12. Biochem Biophys Res Commun 353: 60–66.

Shimono, Y., M. Zabala, R.W. Cho, N. Lobo, P. Dalerba, D. Qian, M. Diehn, H. Liu, S.P. Panula, E. Chiao, F.M. Dirbas, G. Somlo, R.A. Pera, K. Lao, and M.F. Clarke. 2009. Downregulation of miRNA-200c links breast cancer stem cells with normal stem cells. Cell 138: 592–603.

Siclari, V.A., and L. Qin. 2010. Targeting the osteosarcoma cancer stem cell. J Orthop Surg Res 5: 78.

Singh, S.K., I.D.Clarke, M. Terasaki, V.E. Bonn, C. Hawkins, J. Squire, and P.B. Dirks. 2003. Identification of a cancer stem cell in human brain tumors. Cancer Res 63: 5821–5828.

Singh, S.K., I.D. Clarke, T. Hide, and P.B. Dirks. 2004. Cancer stem cells in nervous system tumors. Oncogene. 23: 7267–7273.

Sladek, N.E. 2003. Human aldehyde dehydrogenases: Potential pathological, pharmacological, and toxicological impact. J Biochem Mol Toxicol 17: 7–23.

Song, B., Y. Wang, Y. Xi, K. Kudo, S. Bruheim, G.I. Botchkina, E. Gavin, Y. Wan, A. Formentini, M. Kornmann, O. Fodstad, and J. Ju. 2009. Mechanism of chemoresistance mediated by miR-140 in human osteosarcoma and colon cancer cells. Oncogene 28: 4065–4074.

Stratford, E.W., R. Castro, A. Wennerstrøm, R. Holm, M. Else, L. Silj, B. Bodil, and M. Ola. 2011. Liposarcoma cells with aldefluor and CD133 activity have a cancer stem cell potential Clinical Sarcoma Research 1: 8.

Subbiah, V., P. Anderson, A.J. Lazar, E. Burdett, K. Raymond, and J.A. Ludwig. 2009. Ewing's sarcoma: standard and experimental treatment options. Curr Treat Options Oncol 10: 126–140.

Sulzbacher, I., P. Birner, C. Toma, N. Wick, and P.R. Mazal. 2007. Expression of c-kit in human osteosarcoma and its relevance as a prognostic marker. J Clin Pathol 60: 804–807.

Suvà, M.L., N. Riggi, J.C. Stehle, K. Baumer, S. Tercier, J.M. Joseph, D. Suvà, V. Clément, P. Provero, L. Cironi, M.C. Osterheld, L. Guillou, and I. Stamenkovic. 2009. Identification of cancer stem cells in Ewing's sarcoma. Cancer Res 69: 1776–1781.

Ta, H.T., C.R. Dass, P.F. Choong, and D.E. Dunstan: Osteosarcoma treatment: stateof the art. Cancer Metastasis Rev 28: 247–263.

Tai, M.H., C.C. Chang, L.K. Olson, and J.E. Trosko. 2005. Oct4 expression in adult human stem cells: evidence in support of the stem cell theory of carcinogenesis. Carcinogenesis 26: 495–502.

Takahashi, R., T. Fumitaka, T. Fujiwara, O. Makiko, and T. Ochiya. 2011. Review: Cancer Stem Cells in Breast Cancer. Cancers 3: 1311–1328.

Takebe, N., P.J. Harris, R.Q. Warren, and S.P. Ivy. 2011. Targeting cancer stem cells by inhibiting Wnt, Notch, and Hedgehog pathways. Nat Rev Clin Oncol 8: 97–106.

Tang, Q.L., Y. Liang, X.B. Xie, J.Q. Yin, C.Y. Zou, Z.Q. Zhao, J.N. Shen, and J. Wang. 2011. Enrichment of osteosarcoma stem cells by chemotherapy. Chin J Cancer 30: 426–432.

Tang, Q.L., Z.Q. Zhao, J.C. Li, Y. Liang, J.Q. Yin, C.Y. Zou, X.B. Xie, Y.X. Zeng, J.N. Shen, T. Kang, and J. Wang. 2011. Salinomycin inhibits osteosarcoma by targeting its tumor stem cells. Cancer Lett 311: 113–121.

Taubert, H., T. Greither, D. Kaushal, P. Wurl, M. Bache, F. Bartel, A. Kehlen, C. Lautenschläger, L. Harris, K. Kraemer, A. Meye, M. Kappler, H. Schmidt, H.J. Holzhausen, and S. Hauptmann. 2007. Expression of the stem cell self-renewal geneHiwi and risk of tumor-related death in patients with soft tissuesarcoma. Oncogene 26: 1098–1100.

Taubert, H., P. Wurl, T. Greither, M. Kappler, M. Bache, F. Bartel, A. Kehlen, C. Lautenschlager, L.C. Harris, D. Kaushal, S. Fussel, A. Meye, A. Bohnke, H. Schmidt, H.J. Holzhausen, and S. Hauptmann. 2007. Stem cell-associated genes are extremely poor prognostic factors for soft-tissue sarcoma patients. Oncogene 26: 7170–7174.

Terry, J., and T. Nielsen. 2010. Expression of CD133 in synovial sarcoma. Appl Immunohistochem Mol Morphol 18: 159–165.

Thorne, S.H., W. Liang, P. Sampath, T. Schmidt, R. Sikorski, A. Beilhack, and C.H. Contag. 2010. Targeting localized immune suppression within the tumor through repeat cycles of immune cell-oncolytic virus combination therapy. Mol Ther 18: 1698–1705.

Tirino, V., V. Desiderio, R. d'Aquino, F. De Francesco, G. Pirozzi, A. Graziano, U. Galderisi, C. Cavaliere, A. De Rosa, G. Papaccio, and A. Giordano. 2008. Detection and characterization of CD133+ cancer stem cells in human solid tumors. PLoS One 3: e3469.

Tirino, V., V. Desiderio, F. Paino, A. De Rosa, F. Papaccio, F. Fazioli, G. Pirozzi, and G. Papaccio. 2011. Human primary bone sarcomas contain CD133+ cancer stem cells displaying high tumorigenicity *in vivo*. FASEB J 25: 2022–2030.

Toguchida J. Sarcoma-initiating cells and tissue stem cells. pp. 65–71. In: H. Esumi, N. Takakura, K. Miyazono, M. Mori. [eds.]. 2009. Experimental Medicine. 27. Yodosha, Tokyo, Japan.

Toro, J.R., L.B. Travis, H.J. Wu, K. Zhu, C.D. Fletcher, and S.S. Devesa. 2006. Incidence patterns of soft tissue sarcomas, regardless of primary site, in the surveillance, epidemiology and end results program, 1978–2001: An analysis of 26,758 cases. Int J Cancer 119: 2922–2930.

Tostar, U., R. Toftgård, P.G. Zaphiropoulos, and T. Shimokawa. 2010. Reduction of human embryonal rhabdomyosarcoma tumor growth by inhibition of the hedgehog signaling pathway. Genes Cancer 1: 941–951.

Tsuchida, R., B. Das, H. Yeger, G. Koren, M. Shibuya, P.S. Thorner, S. Baruchel, and D. Malkin. 2008. Cisplatin treatment increases survival and expansion of a highly tumorigenic side-population fraction by upregulating VEGF/Flt1 autocrine signaling. Oncogene 27: 3923–3934.

Tsukahara, T., Y. Nabeta, S. Kawaguchi, H. Ikeda, Y. Sato, K. Shimozawa, K. Ida, H. Asanuma, Y. Hirohashi, T. Torigoe, H. Hiraga, S. Nagoya, T. Wada, T. Yamashita, and N. Sato. 2004. Identification of human autologous cytotoxic T-lymphocyte-defined osteosarcoma gene that encodes a transcriptional regulator, papillomavirus binding factor. Cancer Res 64: 5442–5448.

Tsukahara, T., T. Torigoe, Y. Tamura, T. Wada, S. Kawaguchi, T. Tsuruma, K. Hirata, T. Yamashita, and N. Sato. 2008. Antigenic peptide vaccination: Provoking immune response and clinical benefit for cancer. Curr Immunol Rev 4: 235–241.

Tuan, R.S., G. Boland, and R. Tuli. 2003. Adult mesenchymal stem cells and cell-based tissue engineering. Arthritis Research and Therapy 5: 32–45.

Veselska, R., M. Hermanova, T. Loja, P. Chlapek, I. Zambo, K. Vesely, K. Zitterbart, and J. Sterba. 2008. Nestin expression in osteosarcomas and derivation of nestin/CD133 positive osteosarcoma cell lines. BMC Cancer 8: 300.

Visvader, J.E. 2011. Cells of origin in cancer. Nature 469: 314–322.

Visvader, J.E., and G.J. Lindeman. 2008. Cancer stem cells in solid tumors: accumulating evidence and unresolved questions. Nat Rev Cancer 8: 755–768.

Von Levetzow, C., X. Jiang, Y. Gwye, G. von Levetzow, L. Hung, A. Cooper, J.H. Hsu, and E.R. Lawlor. 2011. Modeling initiation of Ewing sarcoma in human neural crest cells. PLoS One 6: e19305.

Wachtel, M., T. Runge, I. Leuschner, S. Stegmaier, E. Koscielniak, J. Treuner, B. Odermatt, S. Behnke, F.K. Niggli, and B.W. Schäfer. 2006. Subtype and prognostic classification of rhabdomyosarcoma by immunohistochemistry. J Clin Oncol 24: 816–822.

Walter, D., S. Satheesha, P. Albrecht, B.C. Bornhauser, V. D'Alessandro, S.M. Oesch, H. Rehrauer, I. Leuschner, E. Koscielniak, C. Gengler, H. Moch, M. Bernasconi, F.K. Niggli, and B.W. Schäfer. 2011. CD133 positive embryonal rhabdomyosarcoma stem-like cell population is enriched in rhabdospheres. PLoS One 6: e19506.

Wang, C., T. Maass, M. Krupp, F. Thieringer, S. Strand, M.A. Wörns, A.P. Barreiros, P.R. Galle, and A. Teufel. 2009. A systems biology perspective on cholangiocellular carcinoma development: focus on MAPK-signaling and the extracellular environment. J Hepatol 50: 1122–1131.

Wang, L., P. Park, and C.Y. Lin. 2009. Characterization of stem cell attributes in human osteosarcoma cell lines. Cancer Biol Ther 8.

Wang, L., P. Park, H. Zhang, F. La Marca, and C.Y. Lin. 2010. Prospective identification of tumorigenic osteosarcoma cancer stem cells in OS99-1 cells based on high aldehyde dehydrogenase activity. Int J Cancer 128: 294–303.

Warner, J.K., J.C. Wang, K.J. Hope, L. Jin, and J.E. Dick. 2004. Concepts of human leukemic development. Oncogene 23: 7164–7177.

Watt, F.M., S. Estrach, and C.A. Ambler. 2008. Epidermal Notch signalling: differentiation, cancer and adhesion. Curr Opin Cell Biol 20: 171–179.

Wei, H., M.Q. Zhao, W. Dong, Y. Yang, and J.S. Li. 2008. Expression of c-kit proteinand mutational status of the c-kit gene in osteosarcoma and theirclinicopathological significance. J Int Med Res 36: 1008–1014.

Weis, L.D. 1999. The success of limb-salvage surgery in the adolescentpatient with osteogenic sarcoma. Adolesc Med 10: 451–458.

Wesolowski, R., and G.T. Budd. 2010. Use of chemotherapy for patients with bone and soft-tissue sarcomas. Cleve Clin J Med 77 Suppl 1: S23–26.

Wilson, H., M. Huelsmeyer, R. Chun, K.M. Young, K. Friedrichs, and D.J. Argyle. 2008. Isolation and characterization of cancer stem cells from canine osteosarcoma. Vet J 175: 69–75.

Wick, M.R., G.P. Siegal, S.E. Mills, R.C.Thompson, D. Sawhney, and R.E. Fechner. 1987. Dedifferentiated chondrosarcoma of bone. An immunohistochemical and lectin-histochemical study. Virchows Arch A Pathol Anat Histopathol 411: 23–32.

Wu, C., and B.A. Alman. 2008. Side population cells in human cancers. Cancer Lett 268: 1–9.

Wu, C., Q. Wei, V. Utomo, P. Nadesan, H. Whetstone, R. Kandel, J.S. Wunder, and B.A. Alman. 2007. Side population cells isolated from mesenchymal neoplasms have tumor initiating potential. Cancer Res 67: 8216–8222.

Xu, N., T. Papagiannakopoulos, G. Pan, J.A. Thomson, and K.S. Kosik. 2009. MicroRNA-145 regulates OCT4, SOX2, and KLF4 and represses pluripotency in human embryonic stem cells. Cell. 137: 647–658.

Yin, S., J. Li, C. Hu, X. Chen, M. Yao, M. Yan, G. Jiang, C. Ge, H. Xie, D. Wan, S. Yang, S. Zheng, and J. Gu. 2007. CD133 positive hepatocellular carcinoma cells possess high capacity for tumorigenicity. Int J Cancer 120: 1444–1450.

Young, A., J. Lyons, A.L. Miller, V.T. Phan, I.R. Alarcón, and F. McCormick. 2009. Ras signaling and therapies. Adv Cancer Res 102: 1–17.

Yuan, H., N. Corbi, C. Basilico, and L. Dailey. 1995. Developmental-specific activity of the FGF-4 enhancer requires the synergistic action of Sox2 and Oct-3. Genes Dev 9: 2635–2645.

Zamurovic, N., D. Cappellen, D. Rohner, and M. Susa. 2004. Coordinated activation of notch, Wnt, and transforming growth factor-beta signaling pathways in bone morphogenic protein 2-induced osteogenesis. Notch target gene Hey1 inhibits mineralization and Runx2 transcriptional activity. J Biol Chem 279: 37704–37715.

Zeng, W., R. Wan, Y. Zheng, S.R. Singh, and Y. Wei. 2011. Hypoxia, stem cells and bone tumor. Cancer Lett 313: 129–136.

Zhang, P., Y. Yang, P.A. Zweidler-McKay, and D.P. Hughes. 2008. Critical role of notch signaling in osteosarcoma invasion and metastasis. Clin Cancer Res 14: 2962–2969.

Zhou, S., J.D. Schuetz, K.D. Bunting, A.M. Colapietro, J. Sampath, J.J. Morris, I. Lagutina, G.C. Grosveld, M. Osawa, H. Nakauchi, and B.P. Sorrentino. 2001. The ABC transporter Bcrp1/ABCG2 is expressed in a wide variety of stem cells and is a molecular determinant of the side-population phenotype. Nat Med 7: 1028–1034.

THE JOURNAL OF BIOLOGICAL CHEMISTRY VOL. 287, NO. 2, pp. 1397–1405, January 6, 2012

Competitive Interactions of Cancer Cells and Normal Cells via Secretory MicroRNAs*[S]

Received for publication, August 4, 2011, and in revised form, November 23, 2011 Published, JBC Papers in Press, November 28, 2011, DOI 10.1074/jbc.M111.288662

Nobuyoshi Kosaka[‡1], **Haruhisa Iguchi**[‡§1], **Yusuke Yoshioka**[‡2], **Keitaro Hagiwara**[‡¶], **Fumitaka Takeshita**[‡], **and Takahiro Ochiya**[‡3]

From the [‡]*Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, 5-1-1, Tsukiji, Chuo-ku, Tokyo 104-0045, Japan,* [§]*Pharmacology Research Laboratories, Dainippon Sumitomo Pharma Co., Ltd., 1-98, Kasugadenaka 3-chome, Konohana-ku, Osaka 554-0022, Japan, and the* [¶]*Department of Biological Information, Graduate School of Bioscience and Biotechnology, Tokyo Institute of Technology, Yokohama, Kanagawa 226-8501, Japan*

Background: Homeostatic cell competitive system between cancerous cells and non-cancerous cells is considered as the reason for tumor initiation.
Results: Exosomal tumor-suppressive microRNAs secreted by non-cancerous cells inhibit the proliferation of cancerous cells.
Conclusion: Exosomal tumor-suppressive microRNAs act as an inhibitory signal for cancer cells in a cell-competitive process.
Significance: This provides a novel insight into a tumor initiation mechanism.

Normal epithelial cells regulate the secretion of autocrine and paracrine factors that prevent aberrant growth of neighboring cells, leading to healthy development and normal metabolism. One reason for tumor initiation is considered to be a failure of this homeostatic cell competitive system. Here we identify tumor-suppressive microRNAs (miRNAs) secreted by normal cells as anti-proliferative signal entities. Culture supernatant of normal epithelial prostate PNT-2 cells attenuated proliferation of PC-3M-luc cells, prostate cancer cells. Global analysis of miRNA expression signature revealed that a variety of tumor-suppressive miRNAs are released from PNT-2 cells. Of these miRNAs, secretory miR-143 could induce growth inhibition exclusively in cancer cells *in vitro* and *in vivo*. These results suggest that secretory tumor-suppressive miRNAs can act as a death signal in a cell competitive process. This study provides a novel insight into a tumor initiation mechanism.

Competitive interactions among cells are the basis of many homeostatic processes in biology. In *Drosophila*, normal epithelial cells compete with transformed ones for individual survival, which is a process called cell competition (1, 2). If a given group of cells was exposed to some stress, it would be separated into subpopulations of cells with different levels of damage. In noncompetitive conditions, cells with severe damage die in a short time, whereas moderately damaged cells survive to the next generation, indicative of the transduction of a negative phenotype. On the other hand, in competitive conditions even slightly damaged cells are eliminated from the cell group because healthy cells, the "winners," convey death signals to damaged cells, the "losers," and the losers reciprocally confer growth signals to the winners. This feed-forward regulation enables the cell population to eradicate abnormal cells and maintain the same number of normal cells in a limited niche.

Oncogenesis is characterized by genetic and metabolic changes reprogramming living cells to undergo uncontrolled proliferation (3). This suggests that the abnormal cells that are originally destined for elimination can survive and expand against the cell competitive regulation, leading to the formation of a tumor mass. Consistently with this concept, Bondar and Medzhitov (4) showed that the cell competition process involves p53, a tumor-suppressive gene, between the hematopoietic stem cells and progenitor cells, suggesting that gene modifications of p53 could disturb the homeostatic mechanism and give rise to tumor initiation. It is conceivable that p53 target genes could be associated with intercellular communication between winners and losers; however, this literature has not answered the question of whether this regulatory system is mediated by contact-dependent or contact-independent manner. More than 10 years ago a pioneer study suggested that non-cancerous cells co-cultured with cancer cells inhibit the growth of cancer cells *in vitro* (5). This result indicated that humoral factors could be involved in cell competition as intercellular communicators (6).

As recently as a few years ago it was believed that RNAs could not behave as extracellular signal molecules because of their vulnerability to the attack of ribonucleases largely existing in body fluid. Evidence is presently increasing to show that miRNAs[4] contained in exosomes are released from mammalian

* This work was supported in part by a grant-in-aid for the Third-Term Comprehensive 10-Year Strategy for Cancer Control, a grant-in-aid for Scientific Research on Priority Areas Cancer from the Ministry of Education, Culture, Sports, Science, and Technology, the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation, and the Japan Society for the Promotion of Science through the "Funding Program for World-Leading Innovative R&D on Science and Technology (FIRST Program)" initiated by the Council for Science and Technology Policy.
[S] This article contains supplemental Figs. 1–3.
[1] Both authors contributed equally to this work.
[2] A Research Fellow of the Japan Society for the Promotion of Science.
[3] To whom correspondence should be addressed: Division of Molecular and Cellular Medicine, National Cancer Center Research Institute, 1-1, Tsukiji, 5-chome, Chuo-ku, Tokyo 104-0045, Japan. Tel.: 81-3-3542-2511 (ext. 4800); Fax: 81-3-3541-2685; E-mail: tochiya@ncc.go.jp.

[4] The abbreviations used are: miRNA, microRNA; CM, conditioned medium; luc, luciferase; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; QRT-PCR, quantitative real time PCR.

cells and act as a signal transducer (7). It is important that many different tumor-suppressive miRNAs, such as miR-16 and miR-143, are down-regulated in cancer cells, resulting in tumorigenesis, tumor progression, and metastasis (8–11). Taken together, these findings suggest that secretory miRNAs may have favorable aspects for anti-proliferative signals mediating cell competition.

In this report we show that miR-143 expression in normal prostate cells, PNT-2 cells, is higher than that in prostate cancer cells, PC-3M-luc cells, and that miR-143 released from non-cancerous cells transfers growth-inhibitory signals to cancerous cells *in vitro* and *in vivo*. These results suggest that secretory tumor-suppressive miRNAs might be a death signal from winners to losers in the context of cell competition. Secretory miRNAs can be conducive to the maintenance of normal growth and development.

EXPERIMENTAL PROCEDURES

Reagents—Mouse monoclonal anti-KRAS (F234) (sc-30) was purchased from Santa Cruz. Rabbit polyclonal anti-ERK5 (#3372) was purchased from Cell Signaling. Mouse monoclonal anti-actin, clone C4 (MAB1501), was obtained from Millipore. Mouse monoclonal ant-human-CD63 antibody (556019) was purchased from BD Pharmingen. Peroxidase-labeled anti-mouse and anti-rabbit antibodies were included in the Amersham Biosciences ECL PLUS Western blotting Reagents Pack (RPN2124) (GE Healthcare). Synthetic *Caenorhabditis elegans* miRNA cel-miR-39 was synthesized by Qiagen (Valencia, CA). Synthetic hsa-miR-143 (pre-miR-143), the negative control 1 (NC1), has-miR-143 inhibitor molecule (anti-miR-143), and the negative control inhibitor molecule (anti-NC) were purchased from Ambion (Austin, TX). GW4869 was purchased from Calbiochem. Geneticin was purchased from Invitrogen.

Cell Culture—PNT-2 cells, immortalized normal adult prostatic epithelial cell line, were purchased from the DS Pharma Biomedical Co., Ltd. (Osaka, Japan). HEK293 cells, a human embryonic kidney cell line (CRL-1573), were obtained from American Type Culture Collection (Manassas, VA). HEK293 cells were cultured in Dulbecco's modified Eagle's medium containing 10% heat-inactivated fetal bovine serum (FBS) and an antibiotic-antimycotic (Invitrogen) at 37 °C in 5% CO_2. PNT-2 and the prostate cancer cell line, PC-3M-luc cells, continuously expressing firefly luciferase (Xenogen, Alameda, CA), were cultured in RPMI containing 10% heat-inactivated FBS and an antibiotic-antimycotic at 37 °C in 5% CO_2.

Preparation of Conditioned Medium and Exosomes—Before the collection of culture medium, cells were washed 3 times with Advanced RPMI containing an antibiotic-antimycotic and 2 mM L-glutamine (medium A), and the medium was switched to fresh medium A. After incubation for 3 days, medium A was collected and centrifuged at 2000 × *g* for 10 min at room temperature. To thoroughly remove cellular debris, the supernatant was centrifuged again at 12,000 × *g* for 30 min at room temperature or filtered through a 0.22-μm filter (Millipore). The conditioned medium (CM) was then used for miRNA extraction and functional assays as well as exosome isolation.

For exosome preparation the CM was ultracentrifuged at 110,000 × *g* for 70 min at 4 °C. The pellets were washed with 11 ml of PBS, and after ultracentrifugation they were resuspended in PBS. The exosome fraction was measured for its protein content using the Micro BCA Protein Assay kit (Thermo Scientific, Wilmington, DE).

Isolation of MicroRNAs—Isolation of extracellular and cellular miRNAs was performed using the miRNeasy Mini Kit (Qiagen). Two hundred microliters of conditioned medium or cell lysate was diluted with 1 ml of Qiazol Solution. After 5 min of incubation, 10 μl of 0.1 nM cel-miR-39 was added to each aliquot followed by vortexing for 30 s. Subsequent extraction and filter cartridge work were carried out according to the manufacturer's protocol.

Quantitative Real Time PCR (QRT-PCR)—The method for QRT-PCR has been previously described (7). PCR was carried out in 96-well plates using the 7300 Real Time PCR System (Applied Biosystems). All reactions were done in triplicate. All TaqMan MicroRNA Assays were purchased from Applied Biosystems. Cel-miR-39 and RNU6 were used as an invariant control for the CM and cells, respectively.

Immunoblot Analysis—SDS-PAGE gels, SuperSep Ace 5–20% (194–15021) (Wako), were calibrated with Precision Plus Protein Standards (161–0375) (Bio-Rad), and anti-KRAS (1:100), anti-ERK5 (1:1000), anti-CD63 (1:200), and anti-actin (1:1000) were used as primary antibodies. The dilution ratio of each antibody is indicated in parentheses. Two secondary antibodies (peroxidase-labeled anti-mouse and anti-rabbit antibodies) were used at a dilution of 1:10,000. Bound antibodies were visualized by chemiluminescence using the ECL PLUS Western blotting detection System (RPN2132) (GE Healthcare), and luminescent images were analyzed by a LuminoImager (LAS-3000; Fuji Film, Inc.). Only gels for CD63 (BD Biosciences) detection were run under non-reducing conditions.

Plasmids—The primary-miR-143 expression vector was purchased from TaKaRa BIO. For luciferase-based reporter gene assays, pLucNeo was constructed by inserting a firefly luciferase gene derived from the pGL3-control (Promega) into the pEYFP-1 vector (Clontech) at BglII and AflII sites. The sensor vector for miR-143 was constructed by introducing tandem binding sites with perfect complementarity to miR-143 separated by a four-nucleotide spacer into the NotI site of psiCHECK2 (Promega). The sequences of the binding site are as follows: 5′-AAACCTAGAGCGGCCGCGAGCTACAGTGCT***TCATCTC***AAAGAATTCTTGAGCTACAGTGCT***TCATCTC***AGCGGCCGCTGGCCGCAA-3′ (sense) and 5′-TTGCGGCCAGCGGCCGCTGAGATGAAGCACTGTAGCTCAAGAATTCTTTGAGATGAAGCACTGTAGCTCGCGGCCGCTCTAGGTTT-3′ (antisense). The "seed" sequence of miR-143 is indicated by bold italics. In a mutated miR-143 sensor vector, the seed sequence, TCATCTC, was displaced with GACGAGA. All the plasmids were verified by DNA sequencing.

Transient Transfection Assays—Transfections of 10 nM miR-143 mimic and 3 nM anti-miR-143 were accomplished with the DharmaFECT Transfection Reagent (Thermo Scientific) according to the manufacturer's protocol. The total amounts of miRNAs for each transfection were equally adjusted by the addition of NC1 and anti-NC, respectively.

Establishment of Stable Cell Lines—Stable HEK293 cell lines that express miR-143 were generated by selection with 300 μg/ml Geneticin. HEK293 cells were transfected with 0.5 μg of the pri-miR-143 expression vector at 90% confluency in 24-well dishes using a Lipofectamine LTX reagent in accordance with the manufacturer's instructions. Twelve hours after the transfection, the cells were re-plated in a 10-cm dish followed by a 3-week selection with the antibiotic. Ten surviving single colonies were picked up from each transfectant and then cultured for another 2 weeks. The cells expressing the largest amount of miR-143 among transfectants were used as miR-143 stably expressing cells.

Luciferase Reporter Assay—HEK293 cells were cultured at a density of 1×10^4 cells/well in 96-well tissue culture plates overnight, and miRNA transfections or the addition of CM was performed. The cells were harvested, and renilla luciferase activity was measured and normalized by firefly luciferase activity (10). All assays were performed in triplicate and repeated at least three times, and the most representative results are shown.

Cell Growth Assay—PC-3M-luc cells were seeded at a density of 2×10^3 cells/well in a 96-well plate. The following day the cells were transfected with mature miRNAs or incubated with a CM. Twenty-four hours later the culture medium of the transfected cells was switched to medium A, whereas the conditioned medium was not changed. After a 3-day culture, cells were harvested for the measurement of firefly luciferase activity. To know the cellular proliferation by the tetrazolium-based colorimetric MTT assay, 20 μl CM of TetraColor ONE (SEIKAGAKU Corp., Tokyo, Japan) was added to each well after 72 h of culture. After 2–4 h of incubation at 37 °C, the optical density was measured at a wavelength of 450 nm using a microplate reader.

PKH67-labeled Exosome Transfer—Purified exosomes derived from PNT-2 CM were labeled with a PKH67 green fluorescent labeling kit (Sigma). Exosomes were incubated with 2 μM PKH67 for 5 min, washed 4 times using a 100-kDa filter (Microcon YM-100, Millipore) to remove excess dye, and incubated with PC-3M-luc cells at 37 °C.

Co-culture Experiment—In co-culture experiments, 2×10^5 cells/well of PNT-2 cells were plated in 6-well plates. To stain the PNT-2 cells with BODIPY-TR-ceramide (Invitrogen), 5 μM BODIPY-TR-ceramide in a non-serum culture medium was added and incubated with the cells at 37 °C. After 30 min the cells were rinsed several times with a non-serum culture medium and incubated in a fresh medium at 37 °C for an additional 30 min. After the staining of PNT-2 cells by BODIPY-TR-ceramide, labeling of PC-3M-luc cells with PKH67 was performed in accordance with the manufacturer's instructions. After that, labeled PC-3M-luc cells were added and co-cultured with PNT-2 cells for 12 h at 37 °C.

Microarray Analysis—To detect the miRNAs in exosomes and cells derived from PNT-2 and PC-3M-luc cells, 100 ng of total RNA was labeled and hybridized using a human microRNA microarray kit (Agilent Technologies) according to the manufacturer's protocol (Protocol for Use with Agilent MicroRNA Microarrays Version 1.5). Hybridization signals were detected using a DNA microarray scanner (Agilent Technologies), and the scanned images were analyzed using Agilent Feature Extraction software.

Evaluation of Tumor-suppressive miRNA Delivery to Subcutaneously Implanted Prostate Cancer Cell Line in Mice—Animal experiments in this study were performed in compliance with the guidelines of the Institute for Laboratory Animal Research, National Cancer Center Research Institute. Seven-week-old male Balb/c athymic nude mice (CLEA Japan, Shizuoka, Japan) were anesthetized by exposure to 3% isoflurane for injections and *in vivo* imaging. Four days ahead of the first CM injection, the anesthetized animals were subcutaneously injected with 5×10^5 PC-3M-luc cells suspended in 100 μl of sterile Dulbecco's phosphate-buffered saline into each dorsal region. Five hundred μl of CM derived from miR-143-overexpressing HEK293 cells and control cells were daily injected into each tumor from day 0 to 6. For *in vivo* imaging, the mice were administered D-luciferin (150 mg/kg, Promega) by intraperitoneal injection. Ten minutes later, photons from animal whole bodies were counted using the IVIS imaging system (Xenogen) according to the manufacturer's instructions. Data were analyzed using LIVINGIMAGE 2.50 software (Xenogen).

RESULTS

Suppression of Prostate Cancer Cell Proliferation by Conditioned Medium Isolated from Non-cancerous Prostatic Cell—Cell competition is a homeostatic mechanism for the accommodation of an appropriate number of cells in a limited niche or stroma (1). Based on this idea it is possible that the cell competition between normal and abnormal cells frequently occurs in a precancerous state. Of note is that non-cancerous cells suppress cancer cell development by contact-independent interaction (12). For instance, endothelial cells provide the major extracellular heparan sulfate proteoglycan as anti-proliferative signals (12); however, the molecular mechanism by which the other types of cells in a tumor environment associate with cancer cells is not fully understood.

To analyze the mechanism, we treated a hormone-insensitive prostatic carcinoma cell line, PC-3M-luc cells, with a CM from the non-cancerous prostate cell line PNT-2 cells. After a 3-day incubation, the PNT-2 CM inhibited the growth of the PC-3M-luc cells up to ~10% compared with the cell growth treated by fresh culture medium (Fig. 1*A*; compare *lanes 1* and *3*). In contrast, the growth of PC-3M-luc cells incubated in the CM of PC-3M-luc cells themselves showed no inhibitory effect (Fig. 1*A*; compare *lanes 1* and *2*). To determine that the performed treatments did not affect the luciferase activity, we also used the colorimetric MTT assay to measure the cell growth of PC-3M-luc cells. As shown in supplemental Fig. 1*A*, not only luciferase assay but also MTT assay show the inhibition of PC-3M-luc cell proliferation by the addition of PNT-2 cells derived CM, indicating that our treatment did not affect the luciferase activity. These results indicate that the non-cancerous cells may secrete some molecules that can suppress cancer cell proliferation.

In a recent report we showed that miRNAs contained in exosomes are secreted and that their secretion is tightly regulated by neutral sphingomyelinase 2, which is known to hydrolyze sphingomyelins to generate ceramides and trigger the budding