Meeting Report

**Cancer Stem Cells: Constantly Evolving and Functionally Heterogeneous Therapeutic Targets**

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Key words: cancer stem cells, heterogeneity, targeted therapy, drug resistance, metastasis

Running title: 2013 Shanghai International Symposium on Cancer Stem Cells

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Financial support: The symposium was financially sponsored by the Shanghai East Hospital with partial support from Shanghai Jiaotong University and the University of Texas M.D Anderson Cancer Center.

Disclosure of Potential Conflicts of Interest: All authors have no potential conflicts of interest to disclose.
Abstract

Elucidating the origin of and dynamic interrelationship between intratumoral cell subpopulations has clear clinical significance in helping understand the cellular basis of treatment response, therapeutic resistance, and tumor relapse. Cancer stem cells (CSC), together with clonal evolution driven by genetic alterations, generate cancer cell heterogeneity commonly observed in clinical samples. The 2013 Shanghai International Symposium on Cancer Stem Cells brought together leaders in the field to highlight the most recent progress in phenotyping, characterizing, and targeting CSC and in elucidating the relationship between the cell-of-origin of cancer versus CSC. Discussions from the symposium emphasize the urgent need in developing novel therapeutics to target the constantly evolving CSC.

#Footnote: This symposium was held Oct. 17-19, 2013, in Shanghai, China. A complete list of speakers and their presentation titles are provided in Supplementary Table 1. Only part of these presentations is discussed in this Meeting Report.
CSC and cancer cell heterogeneity: Genetic vs. non-genetic

Tumor cell heterogeneity has been appreciated for decades but it is only recently we begin to understand its cellular basis, molecular determinants, and clinical significance. For more than a century, cancer treatment has been largely based on the premise that cancer cells are homogeneously distinct from their normal counterparts and that it is this difference that we need to therapeutically target. We have failed to appreciate that cancer cells themselves are rather heterogeneous and some cancer cells resemble a vitally important population of normal cells, i.e., stem cells (SC), which have the ability to regenerate themselves (i.e., self-renew) and to develop to more mature progeny (i.e., differentiate). Although stem-cell like cancer cells or cancer stem cells (CSC) were experimentally demonstrated as early as 1952 (1), it was not until relatively recently that advances in normal SC research have allowed application of many SC methodologies to studying CSC leading to the revival and explosion of CSC research in the past decade. Both tumor transplantation (2) and lineage tracing (3-5) assays have provided solid evidence for CSC in human and mouse tumors, respectively.

Cancer cell heterogeneity has been traditionally explained by the clonal evolutionary theory. However, recent studies begin to suggest that both clonal evolution driven by the unstable genome of some tumor cell subsets and CSC differentiation driven by epigenetic mechanisms operate hand-in-hand to generate diverse tumor cell types. John E. Dick (University of Toronto) started the symposium by delivering the keynote lecture on genetic and non-genetic determinants of cancer cell heterogeneity, using acute myeloid leukemia (AML) and Ph+ acute lymphoblastic leukemia (ALL) as prime examples.

The leukemia SC (LSC) in AML were first reported to bear the CD34+CD38− marker profile of
normal hematopoietic SC (HSC), with CD34+CD38+ and CD34− fractions containing little clonogenic activity (6). Recent xenotransplantation studies in more severely immune-deficient mice confirm the rarity of LSC but also reveal significant heterogeneity with LSC activity observed in Lin−CD38− fractions as well as CD34+, Lin+, CD38+, and CD45RA+ fractions (7). These observations suggest that the AML LSC do not necessarily always arise from the normal HSC and that multiple CSC subsets with divergent genetic backgrounds may co-exist in a tumorigenic pool, have different origins, and may not be related to one another lineage-wise. Indeed, recent studies in different types of leukemia support that clonal evolution and CSC-directed development may not necessarily be mutually exclusive and may cooperate to create tumor cell heterogeneity. Dick’s work shows that gene signatures specific to AML LSC or normal HSC share a set of genes that defines a common ‘stemness’ program and only this stemness gene signature is a significant independent predictor of AML patient survival (8). Therefore, determinants of stemness influence clinical outcome of AML demonstrating that LSC are clinically relevant and not artifacts of xenotransplantation. The group carried out combined genetic and functional studies of the LSC from AML and B-ALL and the results revealed commonalities between clonal evolution and CSC models of cancer (9). As expected, LSC from diagnostic patient samples are genetically diverse and reconstruction of their genetic ancestry reveals relatedness of multiple subclones of LSC through a complex branching evolutionary process. The discoveries that specific genetic events influence LSC frequency and that genetically distinct LSC evolve through a complex evolutionary process indicate that genetic and functional heterogeneity are closely connected.

The dynamic relationship between genetically diverse tumor cell subclones and functional hierarchy within individual clones is vividly illustrated in the recent study on how clonal repopulation dynamics affect colorectal cancer (CRC) cell response to chemotherapeutic drugs (10). By combining clonal analysis based on DNA copy number alterations (CNA) and
sequencing, lentiviral-mediated lineage marking, and serial tumor transplantations, Kreso et al show that in untreated patient tumors, there exist multiple genetically stable CRC clones of different sizes (dominance). However, within individual genetically identical clones tumor cells are functionally heterogeneous and there is wide variability in different cell lineages with regard to their proliferative kinetics and chemotherapy tolerance. Oxaliplatin eliminates fast-proliferating lineages but enriches previously minor or dormant CRC lineages (10), probably generating new stem cell-like cancer cells resistant to the original chemodrugs.

Dissecting the relationship between CSC vs. the corresponding normal SC and between clonal dynamics vs. intraclonal heterogeneity, and exploring the role of CSC dormancy in therapy resistance are recurring themes of the symposium.

**CSC in glioblastoma multiforme (GBM)**

GBM is the most malignant brain tumor and has among the highest genetic diversity. CSC in GBM have been most commonly enriched using CD133 as a marker although other cell surface molecules such as epidermal growth factor receptor (EGFR) and CD44 have also been used. Transcriptome analysis reveals different gene expression patterns in CD133+ and CD133- GBM cells with the CD133+ population enriched in SC gene signature; however, both cell populations harbor tumorigenic cells and the two seem to have different cells-of-origin (11,12). Luis Parada (UT Southwestern) provided an update on their earlier mouse GBM model studies in which they developed a nestin-ΔTK-IRES-GFP (Nes-ΔTK-GFP) transgenic line that labeled the nestin+ adult neural stem cells in the subventricular zone (3). When this line was crossed with the GBM-prone compound transgenic line (hGFAP-Cre; Nf1fl/+; P53fl/fl; Ptenfl/+), GFP labeled a population of nestin+ mouse GBM cells. When mice were treated with temozolomide (TMZ), a clinically used
chemotherapy drug to treat GBM patients, tumor growth was transiently arrested but later resumed and, remarkably, tumor re-growth was traced to a relatively quiescent population of GFP+ glioma SC (GSC; 3). Simultaneous treatment of mice with both TMZ (to kill fast proliferating bulk GBM cells) and ganciclovir (to eliminate quiescent GSC) led to dramatic tumor growth arrest (3). This study represents one of the first to demonstrate CSC as the cells-of-origin for tumor recurrence in a genetic mouse model. The group is currently performing drug screening against GSC.

GBM manifest prominent intratumoral genetic heterogeneity, i.e., geographically distinct parts of the same tumor may harbor different genetic mutations. A recent deep-sequencing study revealed linear as well as branched patterns of clonal evolution of low-grade gliomas occurring at differing times in the same patient (13). Interestingly, in recurrent tumors (as high-grade gliomas or GBM) without adjuvant chemotherapy, recurrence did not always arise from cells that had the full repertoire of mutations found in the initial tumor (13), implicating the critical importance of non-genetic mechanisms in driving tumor evolution. As expected, treatment with TMZ, which is a mutagen, causes hypermutation in recurrent tumors, a significant number of which harbor driver mutations in the RB and Akt-mTOR pathways (13). Coupled with clonal and cell lineage tracing studies in CRC discussed above (10), these new findings highlight the prominent deficiencies of many currently used chemotherapy drugs in promoting both genetic diversity and de novo CSC generation thus facilitating tumor progression and recurrence.

Peng Huang (M.D Anderson Cancer Center) presented data showing that GSC isolated from xenografts of human GBM cells in mice possess unique energy metabolic characteristics, including low mitochondrial respiration, increased glycolysis for ATP generation and preference for hypoxia to maintain their stemness and tumor-forming capacity (14). Mitochondrial depression in GSC seems to occur mainly at complex II of the electron transport chain with a
down-regulation of the succinate dehydrogenase subunit B, leading to deregulation of hypoxia-inducible factors. Under hypoxia, GSC are resistant to chemotherapeutic agents such as carmustine, but are highly sensitive to glycolytic inhibition. Combination of glycolytic inhibitor 3-bromo-2-oxopropionate-1-propyl ester (3-BrOP) with carmustine exhibited a striking synergistic effect and efficiently killed GSC through a rapid depletion of cellular ATP and inhibition of carmustine-induced DNA repair (14). This drug combination impaired the sphere formation ability of GSC \textit{in vitro} and tumor formation \textit{in vivo}, leading to increased survival of tumor-bearing mice. Mechanistic studies showed that 3-BrOP and carmustine inhibited glyceraldehyde-3-phosphate dehydrogenase and caused a severe energy crisis in GSC (14). These observations suggest that inhibition of glycolysis, in combination with chemotherapy, may be an effective strategy to eradicate GSC.

\section*{Breast CSC (BCSC)}

BCSC are the first CSC prospectively demonstrated in human solid tumors (15) and therefore are among the best characterized. Similar to CSC in other tumor systems, many phenotypic markers (e.g., CD44$^{+}$CD24$^{-/low}$Lin$^{-}$) and strategies (e.g., mammosphere, Aldefluor assay, and side population) have been employed to enrich BCSC, suggesting that BCSC are also heterogeneous. Jenny Chang’s group (The Methodist Hospital, Houston) first showed that BCSC possess intrinsic chemo-resistance (16) and vice versa, residual breast cancer is enriched in BCSC (17). She presented her group’s recent work on BCSC in metastasis. From patient BCSC, a 477-gene tumorigenic signature was generated, among which are \textit{RPL39} and \textit{MLF2}, whose knockdown in patient-derived tumor xenografts could lead to reduced tumor volume and lung metastases with a concomitant decrease in CSC marker expression.
Intriguingly, RNA-Seq analysis revealed mutations in \textit{RPL39} and \textit{MLF2} in 50\% of breast cancer lung metastases. Overexpression of the mutant genes enhanced proliferation, invasion, and self-renewal capacity of BCSC. These studies identify \textit{RPL39} and \textit{MLF2} as novel ‘tumor initiating’ genes that target BCSC and impact lung metastasis.

There has been much debate about BCSC vs. the cell-of-origin of breast cancer. Interestingly, although the BCSC was initially reported to bear CD44\(^+\)CD24\(^-\)Lin\(^-\) phenotype (15), the CD24\(^+\)\text{high}\ and CD24\(^-\)\text{low}\ cells in some patient tumors harbor non-identical genetic alterations suggesting their distinct origins (18). Jane Visvader’s group (Walter and Eliza Hall Institute of Medical Research, Australia) fractionated discrete populations of human mammary epithelial cells that were enriched for mammary basal stem cells (MaSC; CD49\(^f\)EpCAM\(^-\)), luminal progenitors (CD49\(^f\)EpCAM\(^+\)), and mature luminal cells (CD49\(^f\)EpCAM\(^+\)) from normal mammary tissue and preneoplastic specimens of individuals heterozygous for a \textit{BRCA1} mutation. \textit{BRCA1} mutation is clinically linked to the development of basal-like breast cancers. They found that surprisingly, the \textit{BRCA1}-mutant samples display a significant reduction in basal stem cells but a dramatic increase in luminal progenitor cells (19). They further provided evidence that the aberrant luminal progenitor population may represent the transformation target (i.e., cell-of-origin) in \textit{BRCA1}-associated basal-like breast tumors (19). Visvader’s work in mouse models reveals that MaSC are highly responsive to steroid hormones despite lacking expression of the estrogen and progesterone receptors. They have developed novel mouse models to perform lineage tracing and determine the cell-of-origin of specific types of breast cancer.

Numerous intracellular (transcription factors, miRNAs), cell surface (HER2, Notch), and extracellular (cytokines and chemokines) signaling molecules regulate the activity of BCSC and the plasticity of non-BCSC (20). Su-Ling Liu (University of Science and Technology, Hefei,
China) previously demonstrated regulatory roles of miR-93 in normal and malignant breast SC (21). She presented new data that miR-100 expression is related to the cellular differentiation state with lowest expression in cells displaying stem cell markers. Overexpression of miR-100 decreased BCSC and inhibited cancer cell proliferation in vitro and in mouse xenografts by inhibiting Wnt/β-Catenin signaling. Induction of miR-100 expression immediately upon orthotopic implantation or intracardiac injection completely blocked subsequent tumor growth and metastasis formation. Jun-Lin Guan (University of Michigan) talked about the role for focal adhesion kinase (FAK) and its associated signaling pathways in the progression of breast cancer in vivo. Working in mouse models, he showed that inactivation of FAK led to defective BCSC and depletion of the BCSC pool in vivo, leading to reduced mammary tumorigenesis (22).

Richard Pestell’s lab (Thomas Jefferson University, Philadelphia), using knockout and inducible transgenic mouse models, has identified important roles of NF-κB, c-Jun, p21, and DACH1 (Dachshund) in BCSC regulation and cell fate determination (23,24). DACH1, a Forkhead-like nuclear factor, is particularly interesting as it appears to function as a BCSC repressor. Induction of DACH1 expression in vivo reduced CD24low cells in mammary tumors by ~50% and mammospheres by ~60% whereas DACH1 knockdown enhanced mammosphere formation. DACH1 seems to function by binding to the promoters of Sox2 and Nanog repressing their expression. Recently, the group showed that DACH1 also inhibits EMT (epithelial-mesenchymal transition) by repressing Snail translation via inactivating the Y box-binding protein (24).

**Prostate CSC (PCSC)**

Human prostate is a hormone-regulated endocrine organ susceptible to tumor formation,
especially in western countries. Prostatic glands comprise well-demarcated differentiated luminal cells that express markers such as AR (androgen receptor) and PSA (prostate-specific antigen), basal cells that lack expression of differentiation markers but express some SC-associated molecules such as p63, BCL-2, and hTERT, and rare neuroendocrine cells that express certain neural lineage markers. Most prostate cancer (PCa) presents a luminal phenotype, i.e., most PCa cells express AR and PSA. As in many other tumors, two areas of research are intensely pursued and also debated, i.e., the cell-of-origin of PCa (the cells that initiate PCa) and PCSC (the cells that maintain and propagate PCa).

Lineage tracing studies in mouse prostate from Michael Shen (Columbia University) and colleagues have revealed a rare population of luminal prostate epithelial cells that express the homeobox gene *Nkx3.1* (a regulator of prostate epithelial differentiation) resist experimental castration, can regenerate prostate upon androgen re-administration, and, important, can function as an efficient target for oncogenic transformation by *Pten* loss (25). Called CARNs (castration-resistant Nkx3.1⁺ cells), these cells are bipotent and can self-renew *in vivo*. Recent work from the Shen lab shows that deletion of *AR* in CARNs affects their ability to serve as cells-of-origin for PCa in a context-dependent manner - *Pten* deletion with *Kras* activation results in aggressive cancer in the absence of androgen administration in both normal and *AR*-deleted CARNs whereas deletion of *Pten* solely in *AR*-deleted CARNs does not result in tumor formation.

In contrast to the above lineage tracing studies, a tissue recombination assay demonstrates that only purified human prostate basal (i.e., CD49fhiTrop2⁺) but not luminal (CD49fhiTrop2⁻) epithelial cells can be tumorigenically transformed by a combination of ERG, constitutively active AKT, and AR to form adenocarcinomas that histologically resemble the patient tumors (26). These observations support a basal-cell-of-origin of PCa. It is presently unclear why two
independent studies, one in mouse and the other in human, arrive at different conclusions but there could be many interpretations. It could simply be due to the difference between human vs. mouse prostates. It could be related to the differences between the two assays, i.e., in vivo lineage tracing vs. ex vivo tissue recombination. It has been shown that basal cells can manifest increased plasticity when taken out of the prostate and used in in vitro and ex vivo assays (27). Also, most commonly used culture media preferentially support the expansion of basal-like cells. It may also be true that both basal and luminal cells can function as the cells-of-origin of PCa, as already demonstrated by another group (28) but their dynamic ability to do so depends on genetic context and environmental cues (29). Recent lineage tracing studies (28) and tissue regeneration assays (30) begin to converge on the concept that prostatic basal cells can be tumorigenically transformed but progression to and maintenance of overt adenocarcinomas require basal cell differentiation (transition) to luminal cells.

Wei-Qiang Gao’s group identified a basally localized mouse prostate SC population that is Sca1+CD133+CD44+CD117+ (31). Tissue recombination assays demonstrate that a single such cell can regenerate a prostate, although at low frequency. It is unclear whether these cells can function as cells-of-origin of PCa. Gao (Shanghai Jiaotong University) presented ongoing work showing that basal and luminal stem cells in the mouse prostate exhibit different mitotic spindle patterns. Basal stem cells display both symmetric and asymmetric divisions, which lead to different cell fates. During symmetrical divisions, the two daughter cells remain as basal stem cells whereas asymmetrical divisions give rise to one basal stem cell and one luminal cell. In contrast, the luminal cells mainly exhibit symmetrical divisions, which always give rise to two luminal cells. Both luminal and basal stem cells seem to be able to initiate PCa with different division modes. These studies provide further evidence for a hierarchy of epithelial cell lineages during both prostate development and tumorigenesis.
Regardless of PCa cell-of-origin, there exists strong evidence for PCSC or PCa-propagating cells. Dean Tang (M.D Anderson Cancer Center) and his co-workers have recently demonstrated that the undifferentiated (i.e., PSA⁻/⁻) PCa cell population harbors self-renewing long-term tumor-propagating cells that can serially transplant tumors in immune-deficient mice (32). This population preferentially expresses scores of SC-associated and anti-stress genes, remains largely quiescent, and is refractory to chemodrugs, antiandrogens and other stresses. Of clinical significance, the PSA⁻/⁻ PCa cell population, compared to PSA⁺ population, is much more tumorigenic in androgen-ablated hosts and can mediate regeneration of castration-resistant PCa (32). The PSA⁻/⁻ cell population is heterogeneous, containing other more tumorigenic subsets and with ~5-20% PSA⁻/⁻ PCa cells being able to undergo asymmetric cell division regenerating PSA⁺ PCa cells (32). The group also provided evidence that PCSC pool harbors metastasis-initiating cells that can be therapeutically targeted by tumor-suppressive miRNAs such as miR-34a (33).

**CSC in other malignancies**

Hong Wu’s group (UCLA) showed earlier that Pten loss frequently occurs in T-ALL and is associated with therapeutic resistance. Rapamycin, an mTOR inhibitor, was found to suppress leukemia development in Pten null pre-leukemic mice but was insufficient in eliminating leukemia-initiating cells (LIC) after the onset of T-ALL (34). Interestingly, LIC in the Pten-null T-ALL models are actively proliferating, suggesting that they may be sensitive to cell cycle inhibitors. The group then studied two small molecule inhibitors, the Aurora kinase inhibitor VX-680 and the bromodomain inhibitor JQ1, and found that combinatorial treatment of Pten null T-ALL mice with Rapamycin and VX-680, or Rapamycin and JQ1, caused significant elimination of leukemic blasts and diminished the population of LIC. These results highlight synthetic lethality
of co-inhibition of PI3K pathway and cell cycle in Pten null T-ALL LSC.

Multiple myeloma (MM) is a plasma cell malignancy but clonogenic B cells resembling normal memory B cells have been shown to be CSC in MM. Bill Matsui (Johns Hopkins University) presented that the MM CSC can be therapeutically targeted through their phenotypic resemblance to B cells as well as inhibitors of Hedgehog signaling and telomerase. Their recent work demonstrates that GDF15 (growth differentiation factor 15), a TGFβ family member, supports MM CSC within the tumor microenvironment and that circulating levels of this cytokine are associated with the clinical burden of MM CSC and long-term clinical outcome (35).

Jan Paul Medema (University of Amsterdam) presented that high Wnt activity functionally designates the CRC CSC (36). Drug resistance of CRC CSC can be circumvented by pretreatment with histone deacetylase (HDAC) inhibitors, which change the levels of pro- and anti-apoptotic molecules and thereby facilitate cell death. Important, treatment with HDAC inhibitors results in a strong reduction of typical Wnt targets such as Lgr5, and shows strong induction of differentiation. HDAC inhibitors may therefore represent a novel means to sensitize CRC CSC to chemotherapy by enhancing their differentiation. Recent work from Quan Chen (Chinese Academy of Sciences, Beijing) demonstrates that CD44 may be a robust marker for CRC CSC, and osteopontin secreted from macrophages may function as a ligand for CD44 to maintain CSC properties. Interestingly, his group found that cellular prion protein (PrPc) was co-expressed with CD44 in CRC CSC and that the PrPc+ subpopulation within the CD44+ cell population displayed high liver metastatic capability and monoclonal antibodies against PrPc significantly inhibited the tumorigenicity and metastasis of CRC CSCs in models of orthotopic transplantation (37). Early work from Irene Ng (University of Hong Kong) and co-workers demonstrated that in hepatocellular carcinoma (HCC), CD24 is a functional CSC marker that drives HCC through STAT3-mediated Nanog regulation (38). She presented new data showing
that HCC CSC capable of tumor initiation and self-renewal in the presence of chemotherapeutic agents can also be enriched using CD47, a ‘do-not-eat-me’ signal frequently overexpressed in CSC. CD47+ HCC cells secret cathepsin S to regulate CSC activity. Suppression of CD47 by morpholino approach inhibited the growth of HCC in vivo and exerted a chemosensitization effect. These findings shed new light on signaling functions of surface molecules like CD24 and CD47 in hepatocarcinogenesis and provide potential therapeutic targets for HCC patients.

**Cellular reprogramming, cancer cell plasticity, and CSC origin**

Incipient tumors can originate from stem/progenitor cells as well as from the de-differentiation of mature cells. Tumorigenesis, to a certain degree, resembles the somatic cell reprogramming by exogenous (transcription) factors, in which somatic/differentiated cells are turned back to ES cell-like cells. Duanqin Pei (Guangzhou Institute of Biomedicine and Health, China) presented a lecture on the current status of somatic cell reprogramming, focusing on his own studies on the role of vitamin C (Vc) in enhancing reprogramming efficiency by blocking ROS production and promoting cellular demethylations at both H3K36 and H3K9 through histone demethylases Kdm2a/2b and Kdm3/4. The group’s recent work reveals novel functions of Vc in modulating the functions of DNA demethylase TET1 during reprogramming (39). In-depth knowledge of somatic cell reprogramming may offer fresh insight into the tumorigenic transformation.

In established tumors, due to abnormal microenvironment and lack of ‘societal’ control, malignant cells are highly plastic, not only morphologically but also functionally and lineage-wise. Conceivably, CSC can evolve from the cell-of-origin of tumor (i.e., the founding cell that was initially hit by the transforming event) as well as from more mature progeny that has sustained new genetic and epigenetic alterations. Jinsong Liu (M.D Anderson Cancer Center)
presented pathological observations and functional data that polyploidy giant cancer cells (PGCC), frequently observed in clinical samples and increased in advanced, undifferentiated, and relapsed tumors, can generate CSC in culture upon drug or hypoxic treatment (40). Intriguingly, PGCC also seem to have multilineage potential be generating not only cancer cells but also mesenchymal and red blood cells.

**Targeting CSC in the clinic**

The above discussions highlight CSC as constantly evolving and functionally heterogeneous cellular types that distinguish themselves from the bulk cancer cells. The symposium was concluded with a keynote talk by Max Wicha (University of Michigan), who updated the audience on the translational value of targeting CSC in improving personalized and precision cancer therapies. He emphasized a major deficiency in using the primary tumor burden as the major clinical endpoint to measure the outcome of current treatments. Indeed, too often we see dramatic reduction in tumor size but soon patients experience therapy resistance and recurrence without survival benefit. Using breast cancer as a prime example, Wicha illustrated how future clinical trials should be designed by taking into account targeting CSC, which mediate treatment resistance and tumor relapse due to their many unique biological properties. Finally, he discussed potential approaches in targeting CSC, including replacement therapy with tumor-suppressive miRNAs, blocking essential CSC signaling pathways, interfering with the inflammatory microenvironment that supports CSC, and abolishing the CSC self-renewal machinery (41,42). Ultimately, it is envisioned that CSC-targeting therapies can be used in an adjuvant setting or in conjunction with the current therapeutic modalities to achieve long-lasting curing effects, prevent recurrence and metastasis, and prolong patients’ survival.
Acknowledgements

Numerous individuals were involved in the organization and successful completion of this symposium but we would especially like to acknowledge the outstanding assistance from Dr. Xin Wang and her group including Liping (Isabel) Zhang, Jian-Xiong (Irene) He, Zhi-Ping (Linda) Zou, and Hai-Dan (Panny) Lan, all in Shanghai East Hospital, Tongji University School of Medicine.
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Cancer Res Published OnlineFirst April 8, 2014.

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