Commentary

Power of the Eternal Youth

Nanog Expression in the Gestational Choriocarcinoma

Ie-Ming Shih and Kuan-Ting Kuo

From the Department of Pathology, Johns Hopkins Medical Institutions, Baltimore, Maryland

Gestational trophoblastic disease (GTD) is a unique group of lesions because it is derived from the conceptus rather than from the patient. GTD is composed of a spectrum of interrelated disorders that can be broadly divided into three groups¹: i) benign trophoblastic lesions (placental site nodule and exaggerated placental reaction), which are nonneoplastic lesions and are usually incidental findings; ii) hydatidiform moles (complete, partial, and invasive moles), which are aberrant placental derivatives; and iii) gestational trophoblastic neoplasia (GTN) (choriocarcinoma, placental site trophoblastic tumor, and epithelioid trophoblastic tumor), some of which develop from their precursor lesions, ie, complete hydatidiform moles. Choriocarcinoma is the most common type of GTN.

Recent studies have provided new insights into the pathogenesis of GTD and have suggested that various types of trophoblastic lesions are related to the differentiation status of trophoblasts present in the early developing placenta and implantation site.¹ In human placenta, cytotrophoblast is the presumable trophoblastic stem cell that differentiates into either syncytiotrophoblast, which functions as a hormone and pseudovascular tissue, or extravillous (intermediate) trophoblastic cells. The extravillous (intermediate) trophoblastic cells are located in the placental site where the trophoblastic cells infiltrate the decidua, myometrium, and spiral arteries or in the chorion laeve (the fetal membranes) where trophoblastic cells form a cohesive multilayer structure. It has been proposed that after neoplastic transformation of trophoblastic stem cells, presumably the cytotrophoblast, different types of GTD develop along specific differentiation pathways. Choriocarcinoma is composed of variable amounts of neoplastic cytotrophoblast, extravillous (intermediate) trophoblast, and syncytiotrophoblast.² Thus, cytotrophoblast in choriocarcinomas, like its normal counterpart in chorionic villi, undergoes asymmetrical

cell division that results in one daughter cell with either extravillous (intermediate) trophoblastic or syncytiotrophoblastic differentiation whereas the other daughter cell remains as a cytotrophoblast stem cell. In contrast, the neoplastic cytotrophoblastic cells in placental site trophoblastic tumors differentiate mainly into cells resembling implantation site extravillous (intermediate) trophoblastic cells, whereas the neoplastic trophoblastic cells in epithelioid trophoblastic tumors differentiate into extravillous (intermediate) trophoblastic cells similar to those in the chorion laeve. Therefore, choriocarcinoma is the most primitive trophoblastic tumor, and placental site trophoblastic tumor and epithelioid trophoblastic tumor are relatively more differentiated.¹

The clinical outcome in most patients with GTN is excellent after combined chemotherapy and adjuvant surgical procedures. Indeed, choriocarcinoma is among the few human cancers of which metastatic diseases are potentially curable. Unfortunately, some GTN patients develop recurrent tumors after primary treatment. Thus, new therapeutic regimens are needed to minimize the potential chemotherapy-related cytotoxicity and to successfully treat the nonoperable patients. The other clinically challenging issue in managing GTD patients is to predict which patients with complete hydatidiform moles will develop persistent GTD. Available data indicate that 10 to 30% of complete moles develop persistent GTD, as evidenced by a plateau or rise in β -hCG titers or by the presence of extra-uterine invasive hydatidiform mole or choriocarcinoma. Identifying those high-risk patients is an important step to better manage them by using intensive tumor monitoring schemes and introducing early treatment plans. However, reliable prognostic biomarkers have yet to be found. For example, histological grading and expression of cellular proliferation markers such as proliferating cell nuclear antigen and Ki-67 do not show correlation with clinical outcome.^{3,4} Until the fundamental biology of GTN becomes more clearly understood, de-

Accepted for publication July 28, 2008.

Address reprint requests to Ie-Ming Shih, M.D., Ph.D., Department of Pathology, Johns Hopkins Medical Institutions, CRB-2, 1550 Orleans St., Room 305, Baltimore, MD 21231. E-mail: ishih@jhmi.edu.

development of new therapies and discovery of predictive markers will remain empirical.

In this issue of *The American Journal of Pathology*, Cheung and colleagues⁵ described Nanog expression in normal placentas and GTN, providing the functional role of this protein in choriocarcinoma cells. The authors found that Nanog immunoreactivity in early normal placentas was mainly observed in the cytotrophoblast. Nanog expression was only focal and weak in other trophoblastic subpopulations including syncytiotrophoblast, implantation site extravillous (intermediate) trophoblastic cells, and extravillous (intermediate) trophoblastic cells in chorion laeve. In contrast, hydatidiform moles expressed higher levels of Nanog as compared to the first trimester placentas. Interestingly, the investigators found that higher mRNA and protein expression levels of Nanog were associated with worse clinical outcome in hydatidiform moles, ie, increased the risk in developing persistent GTD. Furthermore, the authors observed significantly higher Nanog expression in choriocarcinoma tissues and cell lines than in normal early placentas, suggesting a potential role of Nanog in the development of choriocarcinoma. To examine this possibility, the authors knocked down Nanog using RNA interference and demonstrated that choriocarcinoma cells treated with Nanog shRNA increased apoptosis in a cell culture system. This *in vitro* finding is consistent with their immunostaining results showing an inverse relationship between Nanog immunointensity and expression of M30 (a marker of apoptotic cells) in trophoblastic tissues. Surprisingly, Nanog knock-down had no apparent effects on cellular proliferation in choriocarcinoma cells, suggesting that Nanog is essential to maintain cell survival but may not be sufficient to stimulate cellular proliferation.

The molecular etiology of GTN remains unclear because there are only a few molecular studies available due to its rarity and a lack of experimental models. A new homeobox gene, NECC1, was found to be a potential tumor suppressor in choriocarcinomas.⁶ Expression of NECC1 was decreased in choriocarcinoma cell lines and most choriocarcinoma tissues whereas normal adult tissues and trophoblastic cells in normal placentas expressed abundant NECC1. Ectopic expression of NECC1 in choriocarcinoma cells suppressed tumorigenicity and induced terminal differentiation. Other genes that are potentially involved in the development of choriocarcinoma include Hsp-27,⁷ epidermal growth factor receptor,⁸ DOC-2/hDab2 (a candidate tumor suppressor gene),⁹ and the ras GTPase-activating protein.¹⁰ Epigenetically, down-regulation of *E-cadherin*, *HIC-1*, *p16*, and *TIMP3* resulting from promoter hypermethylation was frequently found in choriocarcinomas, suggesting that reduced expression in those genes may participate in choriocarcinoma development.¹¹ Therefore, the identification and characterization of Nanog overexpression in choriocarcinoma, as demonstrated in this current study, further our understanding of molecular alterations associated with the pathogenesis of choriocarcinoma.

The name Nanog is derived from an Irish mythology, Tir na nOg, which means the land of eternal youth or the land of ever-young. This name is quite pertinent to the

central function of Nanog protein in maintaining the embryonic stem cell as immortal. It also reflects very well the major finding in this study, ie, that Nanog overexpression may equip choriocarcinoma cells with power of the eternal youth, preventing their premature death from apoptosis. Nanog is a member of the homeobox family of DNA-binding transcription factors, and its expression is high in undifferentiated embryonic stem cells and is down-regulated during differentiation (loss of pluripotency) in embryonic stem cells. By comparing gene expression profiles between inner cell mass and trophectoderm in human blastocysts, Adjaye and colleagues¹² identified Nanog as a marker associated with the inner cell mass that has the potential to differentiate into various cell lineages. Transcriptional regulation of Nanog is primarily unknown, but a recent report demonstrated that Kruppel-like factors played a key role in controlling Nanog expression.¹³ Among human neoplasms, diffuse Nanog expression is mainly restricted to germ cell tumors of the testis and many of the tumors harbor a unique structural chromosomal abnormality, isochromosome 12p,¹⁴ where the *Nanog* gene is located. In contrast, expression of Nanog is only detected in a very small percentage of tumor cells as measured by flow cytometry, and Nanog expression has been thought of as one of the defining features for cancer initiating (stem cell-like) cells in several types of human solid tumors, including breast, ovary, and prostate carcinomas.^{15–17}

In view of what has been known about Nanog, the findings from the current study by Cheung and colleagues⁵ have several important biological implications. First, in normal placentas, the robust expression of the stem cell marker Nanog is detected primarily in cytotrophoblastic cells. This observation supports the view that cytotrophoblastic cells represent the trophoblastic stem cells in human placentas. Along this line, Nanog may act in synergism with other nuclear factors such as p63¹⁸ and β -catenin,^{2,19} which are expressed in cytotrophoblastic cells and contribute to their stem cell-like features. Thus, it would be critical to determine whether Nanog protein is responsible to maintain stem-cell like phenotypes in cytotrophoblastic cells and to prevent cytotrophoblastic cells from undergoing differentiation to syncytiotrophoblast and extravillous (intermediate) trophoblastic cells. The identification and characterization of genes expressed in human trophoblast has enhanced our understanding of the lineage and differentiation program of trophoblasts and its relationship to various GTN.^{1,20,21} Thus, the results from the current study are consistent with the view that choriocarcinoma develops as a result of neoplastic transformation from cytotrophoblastic cells in chorionic villi and suggest that the Nanog-expressing cells represent the cancer-initiating/stem cell-like cells in choriocarcinoma. Future studies should assess if Nanog-positive cells from fresh choriocarcinoma tissues are more tumorigenic than Nanog-negative choriocarcinoma cells in a mouse xenograft model. It is well known that the pattern of differentiation in choriocarcinomas recapitulates the stages of early placental development^{2,20,22} in which cytotrophoblastic cells from both the first trimester placenta and choriocarcinoma differentiate into either syncytiotropho-

blast or extravillous (intermediate) trophoblast. It would be interesting to determine whether Nanog expression is indeed confined to neoplastic cytotrophoblast or if it can be found in both neoplastic cytotrophoblast and extravillous (intermediate) trophoblast in choriocarcinomas using the markers that are associated with different trophoblastic subpopulations.^{2,20} Similarly, it remains unclear whether Nanog expression is unique in choriocarcinoma or whether it is also up-regulated in other rare types of GTN including placental site trophoblastic tumor and epithelioid trophoblastic tumor.

Second, the authors described that Nanog knockdown leads to apoptosis in choriocarcinoma cell lines, indicating that Nanog expression may inhibit apoptosis in tumor cells. Thus, in addition to its canonical function in self-renew and maintenance of pluripotency in embryonic stem cells, Nanog may function as an anti-apoptotic factor. This finding is similar to a report showing that knockdown of another embryonic stem cell marker, Oct-4, increased apoptotic activity in embryonic stem cells.²³ Future studies are required to determine the molecular mechanism underlying how Nanog expression confers the anti-apoptotic effect in choriocarcinoma cells.

Third, increased Nanog expression in hydatidiform moles, especially those that progress to persistent GTN, suggests that Nanog may be involved in the development of GTN from a molar placenta and Nanog immunoreactivity could be a useful prognostic marker to distinguish a subset of patients who are at higher risk of developing persistent GTN. We should be mindful, however, based on our past experience, that attempts to predict the behavior of complete hydatidiform moles based on biomarkers are notoriously difficult. The capricious nature of molar pregnancy should never be underestimated. Therefore, larger case-control or perspective cohort studies should be performed to address if Nanog immunoreactivity is an independent prognostic factor and if combination of Nanog with other trophoblastic markers can even better predict the outcome in patients with complete hydatidiform moles.

Finally, the observation that choriocarcinoma cells may have developed molecular dependency on Nanog for cell survival together with a relatively limited distribution of Nanog expression in normal adult tissues promise future development of anti-Nanog therapy in GTN that is refractory to current therapeutic modalities. This is of clinical significance because there are currently only a few known potential molecular targets for GTN.¹ However, several challenges are anticipated before the anti-Nanog target-based therapy becomes a reality. Most importantly, previous experience indicates that targeting nuclear factors such as Nanog would be far more difficult than targeting enzymes and cell surface proteins. It is also critical to ask whether the anti-cancer effects seen in the cell culture system can be recapitulated in patients. Because GTNs are rare diseases and current therapeutic modalities are effective, recruiting a sufficient number of GTN patients in future clinical trials can be certainly challenging.

In summary, the results from the study by Cheung and colleagues⁵ provide new insights into our understanding

of trophoblastic biology and the pathogenesis of GTN, at the same time generating more questions than answers. The current report also underscores the importance in establishing multi-institutional GTD consortiums or collaborative research networks to facilitate sharing of rare GTN specimens and clinical databases. With continued effort, we will be well positioned to unveil further the pathogenesis of this rare but intriguing disease and offer a better diagnostic test and new treatment strategy for GTN patients.

References

- Shih IM: Gestational trophoblastic neoplasms—pathogenesis and potential therapeutic targets. *Lancet Oncol* 2007, 8:642–650
- Mao TL, Kurman RJ, Huang C, Lin MC, Shih I-M: Immunohistochemistry of choriocarcinoma: an aid in differential diagnosis and in elucidating pathogenesis. *Am J Surg Pathol* 2007, 31:1726–1732
- Jeffers MD, Richmond JA, Smith R: Trophoblast proliferation rate does not predict progression to persistent gestational trophoblastic disease in complete hydatidiform mole. *Int J Gynecol Pathol* 1996, 15:34–38
- Cheung AN, Ngan HY, Chen WZ, Loke SL, Collins RJ: The significance of proliferating cell nuclear antigen in human trophoblastic disease: an immunohistochemical study. *Histopathology* 1993, 22:565–568
- Siu MKY, Wong ESY, Chan H-Y, Ngan HYS, Chan KY, Cheung AN: Overexpression of NANOG in gestational trophoblastic diseases—effect on apoptosis, cell invasion, and clinical outcome. *Am J Pathol* 2008, 173:1165–1172
- Asanoma K, Matsuda T, Kondo H, Kato K, Kishino T, Niikawa N, Wake N, Kato H: NECC1, a candidate choriocarcinoma suppressor gene that encodes a homeodomain consensus motif. *Genomics* 2003, 81:15–25
- Garrido C, Ottavi P, Fromentin A, Hammann A, Arrigo AP, Chauffert B, Mehlen P: HSP27 as a mediator of confluence-dependent resistance to cell death induced by anticancer drugs. *Cancer Res* 1997, 57:2661–2667
- Tuncer ZS, Vegh GL, Fulop V, Genest DR, Mok SC, Berkowitz RS: Expression of epidermal growth factor receptor-related family products in gestational trophoblastic diseases and normal placenta and its relationship with development of postmolar tumor. *Gynecol Oncol* 2000, 77:389–393
- Fulop V, Colitti CV, Genest D, Berkowitz RS, Yiu GK, Ng SW, Szepesi J, Mok SC: DOC-2/hDab2, a candidate tumor suppressor gene involved in the development of gestational trophoblastic diseases. *Oncogene* 1998, 17:419–424
- Stähle-Bäckdhal M, Inoue M, Zedenius J, Sandstedt B, DeMarco L, Flam F, Silfversward C, Andrade J, Friedman E: Decreased expression of Ras GTPase activating protein in human trophoblastic tumors. *Am J Pathol* 1995, 146:1073–1078
- Xue WC, Chan KY, Feng HC, Chiu PM, Ngan HY, Tsao SW, Cheung AN: Promoter hypermethylation of multiple genes in hydatidiform mole and choriocarcinoma. *J Mol Diagn* 2004, 6:326–334
- Adajaye J, Huntriss J, Herwig R, BenKahla A, Brink TC, Wierling C, Hultschig C, Groth D, Yaspo ML, Picton HM, Gosden RG, Lehrach H: Primary differentiation in the human blastocyst: comparative molecular portraits of inner cell mass and trophectoderm cells. *Stem Cells* 2005, 23:1514–1525
- Jiang J, Chan YS, Loh YH, Cai J, Tong GQ, Lim CA, Robson P, Zhong S, Ng HH: A core Klf circuitry regulates self-renewal of embryonic stem cells. *Nat Cell Biol* 2008, 10:353–360
- Samaniego F, Rodriguez E, Houldsworth J, Murty VV, Ladanyi M, Lele KP, Chen QG, Dmitrovsky E, Geller NL, Reuter V: Cytogenetic and molecular analysis of human male germ cell tumors: chromosome 12 abnormalities and gene amplification. *Genes Chromosom Cancer* 1990, 1:289–300
- Zhang SBC, Chan MW, Lai HC, Matei D, Schilder JM, Yan PS, Huang TH, Nephew KP: Identification and characterization of ovarian can-

- cer-initiating cells from primary human tumors. *Cancer Res* 2008, 68:4311–4320
16. Ben-Porath I, Thomson MW, Carey VJ, Ge R, Bell GW, Regev A, Weinberg RA: An embryonic stem cell-like gene expression signature in poorly differentiated aggressive human tumors. *Nat Genet* 2008, 40:499–507
17. Gu G, Yuan J, Wills M, Kasper S: Prostate cancer cells with stem cell characteristics reconstitute the original human tumor in vivo. *Cancer Res* 2007, 67:4807–4815
18. Shih IM, Kurman RJ: p63 expression is useful in the distinction of epithelioid trophoblastic and placental site trophoblastic tumors by profiling trophoblastic subpopulations. *Am J Surg Pathol* 2004, 28:1177–1183
19. Li HW, Cheung AN, Tsao SW, Cheung AL: Expression of e-cadherin and beta-catenin in trophoblastic tissue in normal and pathological pregnancies. *Int J Gynecol Pathol* 2003, 22:63–70
20. Shih I-M: Trophogram, an immunohistochemistry-based algorithmic approach, in the differential diagnosis of trophoblastic tumors and tumorlike lesions. *Ann Diagn Pathol* 2007, 11:228–234
21. Shih I-M, Kurman RJ: Molecular basis of gestational trophoblastic diseases. *Curr Mol Med* 2002, 2:1–12
22. Shih I-M, Kurman RJ: Epithelioid trophoblastic tumor—a neoplasm distinct from choriocarcinoma and placental site trophoblastic tumor simulating carcinoma. *Am J Surg Pathol* 1998, 22:1393–1403
23. Guo Y, Mantel C, Hromas RA, Broxmeyer HE: Oct-4 is critical for survival/antiapoptosis of murine embryonic stem cells subjected to stress: effects associated with Stat3/survivin. *Stem Cells* 2008, 26:30–34