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Biomedical advances from tissue culture

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Abstract The demonstration that the “dedifferentiation” of cells commonly observed in the early days of tissue culture was due to selective overgrowth of fibroblasts led to enrichment culture techniques (alternate animal and culture passage) designed to give a selective advantage to functionally differentiated tumor cells. These experiments resulted in the derivation of a large number of functionally differentiated clonal strains of a range of cell types. These results gave rise to the hypothesis that cells in culture accurately represent cells in vivo but without the complex in vivo environment. This concept has been strengthened with the development of hormonally defined culture media in combination with functionally differentiated clonal cell lines, which have augmented the potential of tissue culture studies. The use of hormonally defined media in place of serum-supplemented media demonstrates that hormonal responses and dependencies can be discovered in culture. Discoveries of hormonal dependencies of cancer cells has led to therapies targeting intracellular signaling pathways while discoveries of hormonal responses of pluripotent cells are helping to identify the potential application of stem cells. In these and other ways tissue culture technology will continue to contribute to solving problems of human health.

Keywords Hormonally defined serum-free culture · Erbitux (cetuximab) · Dedifferentiation · Functional cell culture · Growth factors

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Introduction

It is a great honor to be asked to give the 2012 Murakami Memorial lecture. As you may know, Hiroki spent 2 years in my lab learning tissue culture methods and medium development before returning to Japan where he founded the Japanese Association for Animal Cell Technology. Since the membership of JAACT has probably changed with an influx of young members since I gave the Murakami lecture in 2007, I will revisit topics that I believe are still important in animal cell technology.

From the time animal tissue explants could be maintained for extended periods in culture they have been used for medical applications. For example, cultures of chicken, human and monkey cells (Feller et al. 1940; Enders et al. 1949; Youngner et al. 1952)

were used to study the dynamics of viral infection and to develop vaccines for polio, chicken pox, measles and other viral diseases. In addition among the earliest applications of human cell lines such as HeLa cervical adenocarcinoma cells (Scherer et al. 1953) and WI-38 lung fibroblasts (Hayflick et al. 1962) was the propagation of viruses for vaccine development. However, it was not until the functional properties of differentiated cells could be maintained in vitro that tissue culture became a method of studying the physiology and interactions of cells that shed light on their roles in the intact animal. In this paper we will describe some intellectual and technical hurdles that had to be overcome before tissue culture became a widely used "platform technology," and we will provide examples in which basic discoveries from tissue culture have contributed to advances in cancer drug discovery and stem cell biology.

In the late 1950s animal cell cultures rarely exhibited the differentiated properties of their tissue of origin. Usually cells that grew out of explants from different tissues would lose differentiated properties and acquire a common phenotype over time in vitro. Because of this, it was universally believed that cells in culture underwent a process of "dedifferentiation." In other words, cells in culture were different from cells in the intact animal, and thus they could not be used to say anything about the physiology of normal cells in vivo. In 1960 the Sato laboratory did the definitive experiment showing that the lack of differentiated properties in culture was not due to dedifferentiation but to the selection of a minority cell type (connective tissue fibroblasts) in the original tissue sample (Sato et al. 1960). In this experiment freshly isolated mouse liver tissue was divided into three portions. One portion was untreated, another portion was treated with specific anti-liver parenchyma antiserum and complement, and the third portion was treated with anti-fibroblast antiserum and complement. After a brief treatment with anti-liver antiserum and complement, the tissue sample was plated in culture. No effect of this treatment was noted as the subsequent growth was equivalent to the untreated control. When the tissue was treated with anti-fibroblast antiserum, complement fixation was negligible as very few fibroblasts were present in the inoculum, but subsequent cell outgrowth was completely inhibited. Thus, the lack of liver properties in culture was not due to dedifferentiation but to selective overgrowth of liver cells by fibroblasts. Now 50 years after this controversial publication, very few young scientists are aware that dedifferentiation of cells in culture was once dogma.

Once it was clear that differentiated cells were absent from cultures because of fibroblast overgrowth, it was possible to search for solutions to this problem. An enrichment culture protocol that gave differentiated cells a selective advantage over fibroblasts was developed: functionally differentiated animal tumors were alternately passaged through cultures and animals multiple times (Buonassisi et al. 1962). The rationale was that each passage in vitro selected culture-hardy variants of the differentiated tumor cells that were better able to compete with fibroblasts in culture. Initially the Sato laboratory established steroid secreting and ACTH-responsive adrenal tumor cells and ACTH-secreting pituitary tumor cells (Buonassisi et al. 1962). Within 10 years many other functional cell lines had been established with this method including growth hormone-secreting pituitary cells, antigen-specific glial cells, neurotransmitter-synthesizing neuroblastoma cells, differentiating teratoma cells, pigmented melanoma cells, and androgen-secreting testicular cells. The dedifferentiation hypothesis finally was destroyed. It did not die of its own accord as some have written but rather was empirically shown to be false. Furthermore, it was established that cells in culture accurately represented cells in the intact animal suggesting that discoveries made with cells in culture were directly relevant to animal physiology.

While functional differentiated cell lines were being established, compositions of tissue culture media were being optimized by researchers such as Harry Eagle et al. (1956), Puck and Sato (Fisher et al. 1959), and Ham (1965). This research began replacing undefined tissue extracts, blood clots and whole animal sera with amino acids, glucose, small organic molecules, vitamins, trace elements, lipids and purified proteins. However, survival and growth of mammalian cells in these improved media often still required dialyzed serum. Ham and his colleagues contributed greatly to formulating culture media for normal mammalian cells through the 1980s by optimizing the concentrations of medium components (Bettger and Ham 1982). In parallel Sato and his colleagues came to realize that a major function of serum in cell cultures was to provide peptide hormones and growth factors (Sato 1975). This line of

thought led to the formulation of cell type-specific hormonally-defined serum-free media (Barnes and Sato 1980) and to the discovery of novel polypeptide growth factors, which themselves have been incorporated into cell culture media for specific cell types.

Sato and colleagues were prompted to question the role of serum in cell culture by the observation that no existing cell cultures were driven to proliferate by hormones unlike cells in the animal. Therefore the laboratory set out to develop such cultures following the procedure of Biskind and Biskind (1944), who implanted rat ovary tissue in the spleen of ovariectomized rats. The spleen is drained by the hepatic portal vein so any steroids secreted by the implant were destroyed by the liver. The pituitary, sensing a deficiency in steroids, secreted gonadotrophins causing the ovarian implant to grow. Such ovarian cells were established in culture and their growth was found to be dependent on the addition of a crude preparation of leutinizing hormone (LH) provided by the NIH. However, purified LH obtained from another source was ineffective on the ovarian cells. At the same time a post-doctoral fellow in the lab found that NIH-LH was growth stimulatory for 3T3 cells, which suggested a more general activity of a contaminating substance (Armelin 1973). Pursuing this observation, the contaminating activity in NIH-LH was shown to be the novel pituitary-derived growth factor basic FGF (Gospodarowicz et al. 1974). This line of investigation demonstrated that tissue culture could be successfully used as a discovery platform to detect and purify hormone-like factors that were identified as physiologically significant by in vivo experimentation.

Furthermore, this research suggested that the role of serum in cell culture was to provide hormones and hormone-like factors that supported cell viability and proliferation. This hypothesis was confirmed when Hayashi and Sato (1976) showed that GH_3 rat pituitary cells could be grown in medium supplemented with four hormones and transferrin in the complete absence of serum. In subsequent years hormonally defined serum-free media were devised for a wide range of cell lines and cell types. The concept that cells could be grown in a properly formulated hormonally defined medium such that they accurately represent their tissue of origin is not widely recognized among endocrinologists. Nonetheless, it is difficult to deny that the culture approach uncovers novel responses by cell types to hormones and uncovers unknown hormone-like growth factors and cytokines involved in cell–cell communication during development and tissue homeostasis.

Cellular responses discovered in culture should be tested in the animal, and the so-called growth factors should be tested in vivo. This approach can reveal previously unknown complexities, which are necessary to explain essential subtle control mechanisms operating within tissues. Autocrine hormones, for instance, were discovered by the culture approach. In culture, pituitary cells that secrete growth hormone respond to TRH by secreting prolactin. In animals it was shown that TRH causes the secretion of prolactin (Tashjian 1979). Advances in gene manipulation, DNA sequencing technologies, genomic analysis, and proteomics now provide additional means of characterizing the functions of hormone-like growth factors such as PDGF (Antoniades et al. 1979), VEGF (Leung et al. 1989; Keck et al. 1989) and the endocrine FGFs (Yu et al. 2000; Huang et al. 2007) discovered in cell culture experiments.

Hormonally defined media will be central to developing cell-based therapies in the future. The establishment of embryonic stem cells (Evans and Kaufman 1981; Thomson et al. 1998) and induced pluripotent stem cells from re-programmed differentiated cells (Takahashi et al. 2007) have provided a substantial advance, bridging basic tissue culture research and potential clinical applications. However, the clinical potential of these cells depends on an understanding of the environmental factors affecting cell proliferation and differentiation. Much of this understanding will come from the use of serum-free tissue culture. A hormonally defined serum-free medium has been developed that allows the growth of ES or iPS cells without the complicating aspects of serum supplementation and feeder layers (Furue et al. 2008). Other than FGF-2 and insulin the medium does not contain signaling molecules such as BMPs, activin, TGF-β, Wnt, and Nodal that are present in more complicated media to prevent cell differentiation. A similarly simple serum-free medium for human iPS cells has been described recently (Chen et al. 2011). The ability to propagate pluripotent cells in a well-characterized simple environment in the absence of other cell types is an important step towards elucidating the clinical potential of ES and iPS cells.

The concept of hormonally defined media raised the question of whether cancer cells had hormonal

requirements that could be exploited by new therapies (Sato 1980, 1981). At a time when many were focused on identifying cancer-specific antigens that could be targeted by monoclonal antibodies, Sato and colleagues proposed blocking stimulatory hormones with antibodies to their receptors. This now generally accepted idea of targeting growth stimulatory signaling pathways in cancer cells was tested experimentally. As a proof of principle the Sato laboratory made and tested several anti-EGF receptor antibodies with the knowledge that EGF was known to stimulate the growth of a variety of normal and malignant cells and the characterizations of EGF receptors by Stanley Cohen's lab (Cohen et al. 1980). Monoclonal antibodies were deliberately selected that inhibited the binding of EGF to its cell surface receptors (Sato et al. 1983). These neutralizing antibodies to the EGF receptor blocked the growth of A431 cells in culture, and they inhibited the formation of tumors in nude mice (Sato et al. 1983; Masui et al. 1984). This work, which foreshadowed the expansion in research on signaling molecules as cancer drug targets, further confirmed that cells in culture accurately represent cells in vivo. The antibodies were licensed to a biotech company, and a chimeric version of antibody 225, Erbitux (cetuximab), has been approved by the Food and Drug Administration for treating advanced colon cancer and head and neck cancers. The validation of the EGF receptor as a target for cancer therapeutics inspired the development of human monoclonal antibodies (panitumumab) and small molecule kinase inhibitors (gefitinib and erlotinib) that inhibit EGF receptor signaling and are approved to treat colon or lung cancers. A monoclonal antibody (trastuzumab) to the related receptor HER2/*neu* (Hudziak et al. 1989) is approved to treat breast cancer.

The development of Erbitux as a therapy to treat cancer illustrates the conceptual hurdles that had to be overcome for tissue culture to become a successful platform technology. Dedifferentiation of cells in culture did not occur, lines of functionally differentiated cancer cells were established, and they were grown and characterized with respect to their hormone requirements and cell surface receptor expression. Differentiated lines of hybridomas secreting monoclonal antibodies to a hormone receptor were produced, cloned and expanded. These antibodies inhibited the growth of EGR receptor-positive tumor cells in culture, and they inhibited tumor growth in nude mice. The testing over the past 30 years of growth stimulatory responses in vitro of malignant cell lines and tumor types to hormones and growth factors is now paying dividends in the development of experimental drugs targeting proliferative signaling pathways in a variety of cancers. The efficacy of these drugs will be an indicator of how well current tissue culture methods mirror the properties of cell in the body.

References

Antoniades HN, Scher CD, Stiles CD (1979) Purification of human platelet-derived growth factor. Proc Natl Acad Sci USA 7618:1809–1813

Armelin HA (1973) Pituitary extracts and steroid hormones in the control of 3T3 cell growth. Proc Natl Acad Sci USA 70:2702–2706

Barnes D, Sato G (1980) Serum-free cell culture: a unifying approach. Cell 22:649–655

Bettger WJ, Ham RG (1982) The nutrient requirements of cultured mammalian cells. Adv Nutr Res 4:249–286

Biskind MS, Biskind GS (1944) Development of tumors in the rat ovary after transplantation into the spleen. Proc Soc Exp Biol Med 55:176–179

Buonassisi VG, Sato G, Cohen AI (1962) Hormone producing cultures of adrenal and pituitary tumor origin. Proc Natl Acad Sci USA 48:1184–1190

Chen G, Gulbranson DR, Hou Z, Bolin JM, Ruotti V, Probasco MD, Smuga-Otto K, Howden SE, Diol NR, Propson NE, Wagner R, Lee GO, Antosiewicz-Bourget J, Teng JM, Thomson JA (2011) Chemically defined conditions for human iPSC derivation and culture. Nat Methods 8:424–429

Cohen S, Carpenter G, King L (1980) Epidermal growth factor receptor-protein kinase interactions. Co-purification of receptor and epidermal growth factor-enhanced phosphorylation activity. J Biol Chem 255:4834–4842

Eagle H, Oyama VI, Levy M, Horton CL, Fleischman R (1956) The growth response of mammalian cells in tissue culture to L-glutamine and L-glutamic acid. J Biol Chem 218:607–616

Enders JF, Weller TH, Robbins FC (1949) Cultivation of the lansing strain of poliomyelitis virus in cultures of various human embryonic tissues. Science 109:85–87

Evans MJ, Kaufman MH (1981) Establishment in culture of pluripotential cells from mouse embryos. Nature 292:154–156

Feller AE, Enders JF, Weller TH (1940) The prolonged coexistence of vaccinia virus in high titre and living cells in roller tube cultures of chick embryonic tissues. J Exp Med 72:367–388

Fisher HW, Puck TT, Sato G (1959) Molecular growth requirements of single mammalian cells. III. Quantitative colonial growth of single S3 cells in a medium containing synthetic small molecular constituents and two purified protein fractions. J Exp Med 109:649–660

Furue MK, Na J, Jackson JP, Okamoto T, Jones M, Baker D, Hata R, Moore HD, Sato JD, Andrews PW (2008) Heparin promotes the growth of human embryonic stem cells in a defined serum-free medium. Proc Natl Acad Sci USA 105:13409–13414

Gospodarowicz D, Jones KL, Sato G (1974) Purification of a growth factor for ovarian cells from bovine pituitary glands. Proc Natl Acad Sci USA 71:2295–2299

Ham RG (1965) Clonal growth of mammalian cells in a chemically defined, synthetic medium. Proc Natl Acad Sci USA 53:288–293

Hayashi I, Sato GH (1976) Replacement of serum by hormones permits growth of cells in a defined medium. Nature 259:132–134

Hayflick L, Plotkin SA, Norton TW, Koprowski H (1962) Preparation of poliovirus vaccines in a human fetal diploid cell strain. Am J Hyg 75:240–258

Huang X, Yang C, Luo Y, Jin C, Wang F, McKeehan WL (2007) FGFR4 prevents hyperlipidemia and insulin resistance but underlies high-fat diet induced fatty liver. Diabetes 56:2501–2510

Hudziak RM, Lewis GD, Winget M, Fendly BM, Shepard HM, Ullrich A (1989) p185HER2 monoclonal antibody has antiproliferative effects in vitro and sensitizes human breast tumor cells to tumor necrosis factor. Mol Cell Biol 9:1165–1172

Keck P, Hauser SD, Krivi G, Sanzo K, Warren T, Feder J, Connolly DT (1989) Vascular permeability factor, an endothelial cell mitogen related to PDGF. Science 246:1309–1312

Leung DW, Cachianes G, Kuang WJ, Goeddel DW, Ferrara N (1989) Vascular endothelial growth factor is a secreted angiogenic mitogen. Science 246:1306–1309

Masui H, Kawamoto T, Sato JD, Wolf B, Sato G, Mendelsohn J (1984) Growth inhibition of human tumor cells in athymic mice by anti-epidermal growth factor receptor monoclonal antibodies. Cancer Res 44:1002–1007

Sato G (1975) The role of serum in cell culture. In: Litwak G (ed) Biochemical action of hormones, vol VIII. Academic Press, New York, pp 391–396

Sato GH (1980) Towards an endocrine physiology of human cancers. In: Iacobelli S et al (eds) Hormones and cancer. Raven Press, New York, pp 281–285

Sato GH (1981) Antibodies, hormones and cancer. In: Steinberg C, Lefkovits I (eds) The immune system, vol 1. S. Karger, Basel, pp 379–382

Sato G, Zaroff L, Mills SI (1960) Tissue culture populations and their relation to the tissue of origin. Proc Natl Acad Sci USA 46:963–972

Sato JD, Kawamoto T, Le AD, Mendelsohn J, Polikoff J, Sato GH (1983) Biological effects in vitro of monoclonal antibodies to human epidermal growth factors receptors. Mol Biol Med 1:511–529

Scherer WF, Syverton JT, Gey GO (1953) Studies on the propagation in vitro of poliomyelitis viruses. IV. Viral multiplication in a stable strain of human malignant epithelial cells (strain HeLa) derived from an epidermoid carcinoma of the cervix. J Exp Med 97:695–710

Takahashi K, Tanabe K, Ohnuki M, Narita M, Ichisaka T, Tomoda K, Yamanaka S (2007) Induction of pluripotent stem cells from adult human fibroblasts by defined factors. Cell 131:861–872

Tashjian AG (1979) Clonal strains of hormone producing pituitary cells. Meth Enzymol 58:527–535

Thomson JA, Itskovitz-Eldor J, Shapiro SS, Waknitz MA, Swiergiel JJ, Marshall VS, Jones JM (1998) Embryonic stem cell lines derived from human blastocysts. Science 282:1145–1147

Youngner JS, Ward EN, Salk JE (1952) Studies on poliomyelitis viruses in cultures of monkey testicular tissue. I. Propagation of virus in roller tubes. Am J Hyg 55:291–300

Yu C, Wang F, Kan M, Jin C, Jones RB, Weinstein M, Deng CX, McKeehan WL (2000) Elevated cholesterol metabolism and bile acid synthesis in mice lacking membrane tyrosine kinase receptor FGFR4. J Biol Chem 275:15482–15489