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Wacław Szybalski's contribution to immunotherapy: HGPRT mutation & HAT selection as first steps to gene therapy and hybrid techniques in mammalian cells

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ABSTRACT

In this report we describe Wacław Szybalski's fundamental contribution to gene therapy and immunotherapy. His 1962 PNAS paper (Szybalska and Szybalski, 1962) documented the first successful gene repair in mammalian cells. Furthermore, this was also the first report on the HAT selection method used later in many applications. Most importantly, somatic cell fusion and HAT selection were subsequently used to develop monoclonal antibody technology, which contributed significantly to the progress of today's medicine.

When he started to work with eukaryotic cells, Prof. Wacław Szybalski's initial motivation was to study the eukaryotic DNA replication. A preliminary step of this research was checking if halogenated thymidine analogues can selectively increase radiosensitivity of human cells, similarly to bacteria, in order to establish a new mode of cancer therapy (Szybalski, 1992). Eventually, these studies led him to perform the first successful genetic modification of human cells, and more importantly to the stable modification which resulted in the transfer of a given external biochemical trait to daughter cells along cell divisions. This goal was based on a series of experiments designed to identify the useful stable cell lines and to develop highly quantitative methods to study mutations. Further isolation of well-defined mutants helped in constructing proper selection systems (Szybalski, 1992). These pioneering technological advances were to be applied to study mutagens/carcinogens, search for genetic exchange systems, genetic recombination and genetic transformation of human cells. As the 1960s were not the era of disposable plastic dishes, commercial CO2 incubators, defined media for eukaryotic cell lines, this kind of research project required excellent and versatile skills, governed by an open and daring mind.

The project was initiated in 1956 and the following few years spent at the Rutgers University and then at University of Wisconsin brought about several technical advancements in methodology of genetic studies on human cells, such as selective systems for measuring forward and reverse mutation rates (Szybalski, 1959; Szybalski and Szybalska, 1961), generation of chemoresistant mutants of human cell lines (Szybalski and Smith, 1959a; Szybalski et al., 1961) and the beginning of eukaryotic cell radiobiology research (Djordjevic and Szybalski, 1960; Erikson and Szybalski, 1961; Szybalski and Djordjevic, 1960; Szybalski and Smith, 1959b) as well as genetic modification of mammalian cell lines with DNA (Djordjevic and Szybalski, 1960; Szybalski et al., 1960). Finally, in 1962 Wacław Szybalski and his wife Barbara published their most important pioneering study, showing for the first time to the global scientific community how a foreign gene can be introduced into the genome of mammalian cells and providing an elegant way to demonstrate it (Szybalska and Szybalski, 1962). This report brought to the public the first well-documented genetic transformation of human cells correcting the functioning of a metabolic pathway. Thus, it was the first documented case of cellular „gene therapy" by the *gene correction approach*, which we believe strongly, thanks to the pioneering scientific attitude of Wacław Szybalski, made it possible to recently celebrate 50 years of the gene therapy concept. Additionally, this paper provided description of the invention of a novel selection method of transformed mammalian cells, used later in many other applications, most importantly the monoclonal antibody technique.

The HAT selection method designed, used and presented in the 1962 paper was based on the knowledge acquired by Wacław Szybalski and his wife in collaboration with R. Wallace Brockman (Szybalski et al., 1961) on the biochemistry of purine metabolism, and especially on the function of hypoxanthine-guanine phosphoribosyl transferase (HPRT, HPGRT, EC 2.4.2.8, known earlier as IMPPase), which mainly

Abbreviations: ADA-SCID, adenosine deaminase-deficient severe combined immunodeficiency; DNAse, deoxyribonuclease; ES cells, embryonic stem cells; HAT, hypoxanthine, aminopterin, thymidine; HPRT, hypoxanthine-guanine phosphoribosyl transferase; IMP, inosine monophosphate; RT-PCR, reverse transcription polymerase chain reaction.

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transforms hypoxanthine to inosine monophosphate, IMP. In the cells this enzyme functions to synthesize purines from degraded DNA. When *de novo* synthesis of purines is blocked, HPRT would constitute a pathway securing purine synthesis. For this type of selection Szybalski designed a specific HAT medium containing hypoxanthine, aminopterin and thymidine (Szybalski and Szybalska, 1961). Aminopterin is a compound that inhibits de novo purine biosynthesis, so for a cell to survive in its presence, it must scavenge for purines from other compounds. The presence of thymidine allows cells to escape second metabolic block induced by aminopterin and hypoxanthine is the sole purine source enabling the cells with functional HPRT to use it in order to scavenge purines needed for DNA synthesis and continued cell growth.

In this study, several variants of D98 human bone marrow cells were used, which were derived from D98S cell line and termed as: D98/AG, resistant to 8-azaguanine, or D98/AH and D98/AH-2 — sublines of D98/AG resistant also to 8-azahypoxanthine. The 1962 paper describes experiments, in which DNA was isolated from donor D98S and D98/AG cells. These cells were HPRT positive and could grow on the selective HAT medium because they could use hypoxanthine as the sole purine source. The isolated DNA was then used to transform D98/AH or D98/AH-2 cells recipient cells, which were HRPT − (HPRT defective) and could not grow on this medium. However, after transformation using isolated DNA in calcium phosphate buffer (the first highly efficient transformation protocol for mammalian cells, still used to today) (Szybalski and Joannes, 1962), D98/AH or D98/AH-2 cells were able to form colonies, so they had to take up the donor DNA and incorporate it into their genome. Furthermore, incorporated DNA was passed to dividing daughter cells forming clones showing that they were able to withstand the selection pressure. The possibility of spontaneous reversion of the *loss of function* phenotype was also taken under the consideration, however it was thought not to be likely due to: 1/the very low frequency of such event for the HRPT gene (1 out of 10 million or no detectable for the D98/AH-2 cells), 2/the linear relationship between the amount of DNA used and the frequency of transformation events on HAT medium as well as the fact that 3/when DNA isolated from donor HPRT positive cells was treated with DNase before transformation, no colonies were formed. Thus, the authors demonstrated that a *loss of function* feature of a human cell could be restored by introduction of a foreign, functional DNA, and that the restored phenotype can be transmitted to descendant cells and can be therefore stable.

The 1962 work of Szybalski should be remembered for at least two main reasons. It was the first broadly published report on HAT selection method (and not just a conference publication) and the first successful approach of stable genetic modification of mammalian cells. One should add that when a new wave of reports on genetic modifications of human cells was published in the late 70s, most scientists used the HAT selection method. Although many new approaches have been developed since then to isolate and select genetically modified mammalian cells, the initial impulse for this technology came from Wacław Szybalski's discovery.

Indeed, the use of HPRT gene as a selection marker is still popular today. The HPRT minigene has an advantage over other selection cassettes, because one may select for either the loss of HPRT function (8-azahypoxanthine or 6-thioguanine resistance) or its gain in HAT medium allowing for both forward and reverse selection (Stout and Caskey, 1985). Its expression levels are so stable that it is a popular reference gene for quantitative RT-PCR for both normal (Żyżyńska-Granica and Koziak, 2012) and tumor tissues (de Kok et al., 2005) as well as stem cells both human (Chooi et al., in press) and murine (Veazey and Golding, 2011). It makes it a very good selection marker and an indispensable tool in mutagen testing, hybridoma, somatic cell hybrid techniques and even in animal transgenesis, where it is used both as a selection marker (Plagge et al., 1999) and a locus to be modified (Aizawa et al., 2012; Cadieux et al., 2006).

The HPRT gene is X-linked, hemizygous in male cells and functionally hemizygous in female ones, therefore seems to be especially useful for examination of recessive mutations and is often used as a mutation reporter gene due to the ease of mutant's selection (Albertini, 2001). Additionally, a small intron separates exons 1 and 2 from exons 3–8 providing sequences flexible to manipulation (Stout and Caskey, 1985). HPRT minigene cassette is used as a split gene cassette (split into two parts inside an intron sequence) coding for a selection marker for the purpose of *knock-in* detection where the *knocked-in* locus and an introduced sequence have both separate parts of HPRT minigene (Plagge et al., 1999). This approach enables high-throughput *knock-in* in embryonic stem cells (Kim et al., 2008) and an easy selection method for both the presence of selective marker and its lack. Some *knock-in* applications aiming at introduction of new genes or modified genes into a defined locus require the insertion of recombination sites into this locus before the insertion of the chosen transgene. The HPRT locus, coding for a non-essential enzyme if purines can be provided by *de novo* synthesis, is one of the places of choice for gene targeting via homologous recombination in embryonic stem cells both murine (Cadieux et al., 2006) and human as well as human-induced pluripotent stem cells (Aizawa et al., 2012). The usefulness of the HPRT locus for gene targeting is exceptional as it was demonstrated that the frequency of gene targeting in this locus is insensitive to the length of non-homologous DNA transferred to the target chromosomal sequence (Mansour et al., 1990). In one of the newer applications the HPRT locus is used for the rapid and efficient recombination system in which an inducible *cre* minigene floxed by heterologous *loxP* sites is placed in HPRT locus and then replaced with an incoming transgene using Cre recombination (Iacovino et al., 2011). Another application with the high future potential is the use of helper-dependent adenoviral vectors to correct an insertional mutation in the hypoxanthine phosphoribosyl transferase locus using homologous recombination in embryonic stem cells. Comparably high frequency of homologous recombination (0.2%) suggests, that it could be an efficient and relatively safe option for *ex vivo* gene therapy, especially compared with the use of retroviral vectors with their high risk of insertional mutagenesis (Ohbayashi et al., 2005).

HPRT gene is also popular in work with artificial chromosomes as a selection marker (Cheung et al., 2012; Moralli et al., 2006), locus for gene targeting (Wada et al., 1994) and as a split HPRT minigene cassette in chromosome engineering, where it is used for the selection of recombinant chromosomes in mammalian cells (Zheng et al., 2000), even in the formation of human artificial chromosomes (Yamaguchi et al., 2011). It makes HPRT gene both a powerful tool for functional genomic studies, especially with the use of many HPRT-negative cell lines and mouse strains, and a highly promising gene delivery tool in human gene therapy.

Szybalski's 1962 work with HPRT was the first documented case of cellular „gene therapy" by the *gene correction approach* resulting in the stable modification of a metabolic pathway (Szybalska and Szybalski, 1962). It started the work on the first candidate for initial attempts at somatic cell gene therapy: Lesch–Nyhan syndrome, a devastating neurological disease caused by the HPRT deficiency (Miller et al., 1984; Palella et al., 1989). The resulting problems with the assessment of the metabolic pathway's correction led to the development of the first mouse model of a human disease: HPRT-deficient mice (Hooper et al., 1987; Kuehn et al., 1987) and later to the first successful trial of *ex vivo* gene therapy in 1990 for ADA-SCID, disease caused by adenosine deaminase deficiency — another enzyme of purine metabolism (Sheridan, 2011). Since then, over 1,700 clinical trials for gene therapy have been conducted.

Szybalski was also the first to use HAT selection method to identify genetic recombinants following fusion of somatic cells (Krooth, 1964). He tried to fuse D98/AH-2 cells with his own skin cells and generate hybrids with the help of UV-inactivated Sendai virus. Although not pursued by him personally, this line of experiments led to the first important citations of the 1962 paper (Littlefield, 1964), and to the exploration of somatic cell hybrids in various areas of cell biology, including the recently uncovered and highly important —