Chapter 8

Authenticating Hybrid Cell Lines

Raymond W. Nims, Amanda Capes-Davis, Christopher Korch and Yvonne A. Reid

Additional information is available at the end of the chapter

http://dx.doi.org/10.5772/intechopen.80669

Abstract

Hybrid (both intra-species and inter-species) cell lines arise through intentional or non-intentional fusion of somatic cells having different origins. Hybrid cell lines can pose a problem for authentication testing to confirm cell line identity, since the results obtained may not conform to the results expected for the two parental cell types. Thus, depending on the identity testing methodology, a hybrid cell may display characteristics of one of the parental cell type or of both. In some instances, the hybrid cell line may display characteristics that are different from those displayed by either parental cell type; these differences may not necessarily indicate cellular cross-contamination. Testing should be performed as soon as possible after an intended fusion has occurred, so that a baseline reference profile is available for later comparison. In this article, we describe the various approaches that have been used for identifying hybrid cell lines and the results that might be expected when using various technologies for this purpose.

Keywords: cell fusion, hybrid cell line, authentication, immunostaining, isoenzyme analysis, karyotyping, STR profiling, SNP profiling

1. Introduction

Fusion of cells occurs normally in vivo, such as of muscle cells, bone cells, macrophages, during fertilization of germ cells, and in placenta formation [1]. Somatic cell hybrid cell lines (or more simply hybrid cell lines) are cell lines that arise through intentional or nonintentional fusion of somatic cells having different origins [2]. Intra-species and inter-species (cross-species) cell fusions have been described since the 1950s [3] and can occur either spontaneously or can be mediated by human oncogenic viruses (such as Sendai virus, Epstein-Barr virus, human papilloma viruses, hepatitis B and C viruses, human T-cell lymphotropic virus type 1

(HTLV-1), herpesviruses-8/Kaposi sarcoma herpesvirus (HHV-8/KSHV)) [4, 5], polyethylene glycol [6], or electrical pulses (electrofusion) [7–12], resulting in viable syncytial cells (giant cells or polykaryotes) with hybrid genotypes, namely heterokaryons. Mouse-human hetero-hybridoma technology has advanced significantly with the use of electrofusion technology [13]. Currently, electrically induced cell fusion is also being used to develop cancer cells with increased immunogenicity by fusion with dendritic cells for development of anti-tumor vaccines [14, 15].

Cell fusion can be important in the establishment and evolution of cell lines (e.g., [16]) and can lead to cancer progression and metastasis via genetic instability [17–20]. Hybrid cell lines also can arise spontaneously. Numerous examples have been documented [21], including a case where a patient-derived xenograft model underwent spontaneous fusion with normal mouse stromal cells, forming a hybrid cell that was more tumorigenic than the parental lines [22]. Spontaneous cell-cell fusion can act as a mechanism for DNA exchange between malignant and non-malignant cells and for horizontal transmission of malignancy [21, 23]. Spontaneous cell-cell fusion can be challenging to detect. Some cases are only detected incidentally—for example, when unexpected chromosomes are detected during cytogenetic analysis [24].

Intentionally created hybrid cell lines have been used for a variety of purposes, including monoclonal antibody production by mouse × mouse and mouse × human hybridomas [25], gene mapping studies [26], studies of gene expression [27], study of cancer initiation, progression, and metastasis [21, 23, 24, 28, 29], evaluation of drug resistance mechanisms [30]; as well as in the field of virology [31, 32]. Perhaps the most commonly employed inter-species hybrid cell lines, currently, are mouse × human somatic cell hybrids. Examples of intra-species cell hybrids might include mouse × mouse (inter-strain) hybrid cells [33] or hybridomas created by fusion of mouse splenic cells and mouse myeloma cells [34].

Hybrid cell lines can be challenging to authenticate and to confirm that they are valid research models. This paper reviews historical and more recent technologies that have played a role in the authentication of inter-species and intra-species hybrid cell lines. As part of cell line authentication, the identity of a cell line is expected to be established to the species level, or if possible, to the individual donor level. There are a variety of approaches that may be used for this purpose. Over the years, these have included isoenzyme, cytogenetic, and immunological analyses, and more recently, a variety of molecular methods such as restriction length fragment polymorphism (RFLP), nested PCR analysis of mitochondrial genes, short tandem repeat (STR) profiling, single nucleotide polymorphism (SNP) profiling, sequence-based human leukocyte antigen (HLA) typing, and next generation sequencing. Each of these approaches may also be applicable to the authentication of intra-species or inter-species hybrid cell lines, although the results to be expected and, therefore, the interpretation of such results in arriving at the identification of the cell line may differ from those for non-hybrid cell lines. Inter-species hybrid cell lines have a propensity to lose chromosomes during continued passage of the cultures [18]. Such loss occurs especially in the case of hybrids of human and rodent cells, such as human × mouse or human × rat hybrids. In these cases, the human chromosomes tend to be lost with continued passage of the cultures. The results expected to be obtained with several of the authentication techniques mentioned below tend, therefore, to evolve over time as the hybrid cells are cultured [35–38]. This is especially true in the case of karyotyping and total DNA content, but also may impact isoenzyme analysis and molecular-based methods.

2. Methodologies for authenticating hybrid cell lines

2.1. Isoenzyme analysis

Isoenzyme analysis was one of the first methods to be used (as early as 1970s) for determining the species-level identity of cell lines. This method is still being used [39], despite the fact that reagents for performance of the method are not commercially available. There is a considerable amount of historical data, in the public domain, for non-hybrid and hybrid cell lines, therefore, discussion of these results has relevance in deciphering hybrid cell line identities.

In isoenzyme analysis, the gel electrophoresis banding patterns and relative migration distances of intracellular enzyme isoforms are used to confirm the expected animal species of origin for test cells. Normalized migration distances obtained for the set of enzymes evaluated are compared to a set of tabular values for various animal species, and through a process of elimination, the most likely animal species of origin for the test cell is determined. Although the results of isoenzyme analysis historically have been used to confirm species-level (intra-species) identity of a cell line, the method also can be used to demonstrate the existence of an inter-species cell mixture [40] or to authenticate inter-species hybrid cell lines [27, 31–32, 36–38, 41, 42].

When evaluating inter-species cell mixtures using isoenzyme analysis, bands migrating as expected for each of the parental species comprising the mixture are observed, provided that a sufficient percentage of cells of both species are present in the mixture [31, 38–41]. In the case of inter-species hybrid cell lines, however, a variety of possible outcomes may be obtained when authenticating using isoenzyme analysis. These outcomes might include, for instance, bands for certain enzymes that migrate as expected for both parental species or for only one of the two parental species, or bands that migrate differently than expected for either parental species (**Figure 1**).

As is evident from **Figure 1**, interpretation of an isoenzyme analysis electropherogram for a hybrid cell is not as straightforward as it is for a cell mixture. The chromosomes contributed to the hybrid cell by the two parental cells determine the outcome of the isoenzyme analysis results for any given enzyme, as the genes encoding the enzymes evaluated in this method are scattered among the various chromosomes of the various animal species [37]. In fact, isoenzyme analysis was performed commonly in early gene mapping studies because linkage between genes encoding an isoenzyme and a gene of interest could be used to assign the chromosomal location for the gene of interest. Due to uncertainty of the assortment of parental chromosomes (and encoded enzyme genes) into a hybrid cell, it is not possible to predict in advance the phenotype and, therefore, the electrophoretic characteristics of enzymes being evaluated using isoenzyme analysis. This is depicted well by the results of authentication of a series of human × bovine hybrid cell lines (**Table 1**) by van Olphen and Mittal [32].

Authenticating an intentionally created hybrid cell line using isoenzyme analysis, therefore, entails evaluation of the hybrid as soon as possible after fusion of the parental cells. The migration patterns displayed by the enzymes evaluated are then considered to be the reference pattern to be expected for the hybrid cell during subsequent authentication assays. This is similar to the case for DNA fingerprinting. When reviewing historical data of cell line

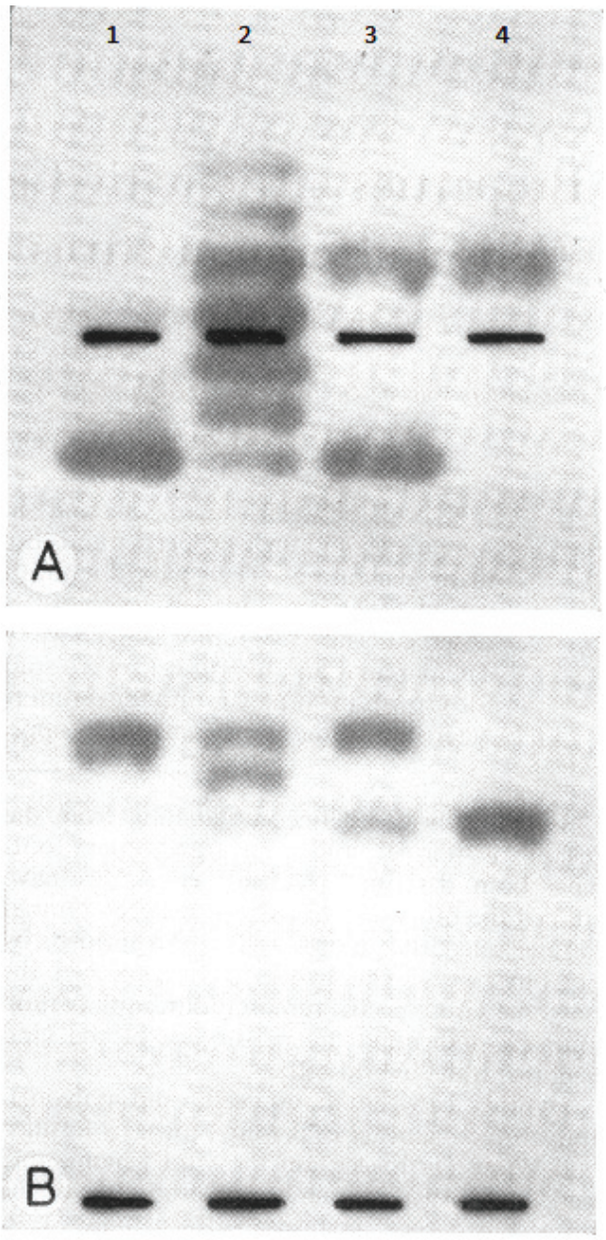


Figure 1. Isoenzyme analysis of (A) lactate dehydrogenase; and (B) 6-phosphogluconate dehydrogenase in parental cells, a cell mixture, and a hybrid cell. Lane 1: parental rat-SV40 cell; lane 2: hybrid H3 (rat-SV40 × mouse 3T3 TK$^-$); lane 3: mixture of rat-SV40 and mouse 3T3 TK$^-$; lane 4: parental mouse 3T3 TK$^-$ (from [31]). The black lines in each lane indicate the origins (the slots in the wells into which the protein is loaded).

authentication by isoenzyme analysis, allowance must be made for loss of parental chromosomes during extended culture, as this may result in loss of electrophoretic bands associated with certain enzyme isoforms over time. Reagents for performing isoenzyme analysis are no longer commercially available, so other methods to be described below are now more commonly being used for hybrid cell authentication.

2.2. Immunostaining for surface antigens

The species-level identification of cells through use of antisera directed against species-specific cell surface markers has also been applied to the authentication of inter-species cell hybrids [32].

Method	Parental 293-Puro	Parental MDBK-Neo	Hybrid cell line		
			BHH2C	BHH3	BHH8
Immunofluorescent staining for surface antigen	Human	Bovine	Human	Bovine	Bovine
Karyotyping					
Number of human chromosomes	62	0	71	27	19
Number of bovine chromosomes	0	60	4	48	47
Total number of chromosomes[a]	62	60	97	113	103
Isoenzyme analysis					
Glucose-6-phosphate dehydrogenase	Human	Bovine	Human	Bovine +[b]	Bovine +
Malate dehydrogenase	Human	Bovine	Human	Bovine +	Bovine +
Lactate dehydrogenase	Human	Bovine	Human +[c]	Bovine +	Bovine +
Nucleoside phosphorylase	Human	Bovine	Human	Bovine	Bovine
Flow cytometry					
Total DNA content	171.1 ± 5.3	166.0 ± 4.9[d]	254.7 ± 6.8	297.7 ± 10.3	288.6 ± 7.5

[a]Total chromosomes includes human, bovine, and unidentified chromosomes.
[b]Bands expected for bovine were observed, along with extra bands.
[c]Bands expected for human were observed, along with extra bands.
[d]Mean ± standard deviation, units are relative DNA content.

Table 1. Authentication results for three hybrid (human × bovine) cell lines (data from [32]).

In the case of an inter-species cell mixture, immunostaining reagents directed against conserved surface antigens of each parental species would each be expected to demonstrate reactivity. In the case of inter-species hybrids, the result that might be obtained is not so easily predicted in advance. For instance, as shown in **Table 1**, the surface antigens that are actually detected in a set of hybrids may be derived from only one or the other of the parental species (**Figure 2**).

It is also possible, depending upon the species-specific antisera employed and the chromosomal make-up of the hybrid cell, for an inter-species hybrid cell to display surface staining for antigens of both parental species. For instance, Kano et al. [43] reported that all human × mouse hybrids evaluated in their study displayed mouse surface antigens, while most but not all also displayed human surface antigens. Surface antigens characteristic of both parental species were displayed by all human × hamster and human × rat hybrid cells evaluated. Gallagher et al. [44] reported similar results in their analysis of the surface antigens in human [HeLa] × mouse hybrid [3T3.4E] cells. Surface antigens characteristic of both parental cells were displayed by the hybrid cells.

It is also possible that by the staining of interspecies hybrid cells, one may detect a surface antigen that is not expressed by either parental cell. For instance, van Someren et al. [41]

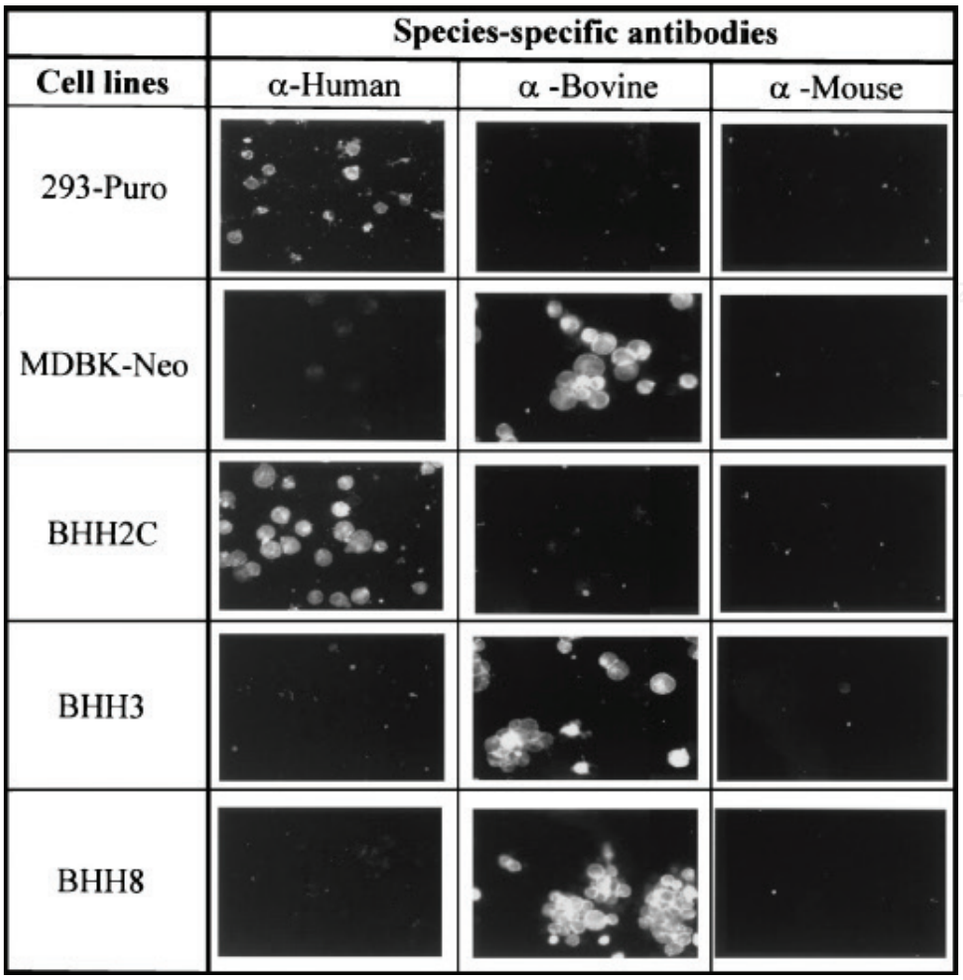


Figure 2. Use of immunostaining against species-specific surface antigens to characterize parental human [293-Puro] and bovine [MDBK-Neo] cells, and three human × bovine hybrid cell lines [BHH2C, BHH3, and BHH8]. Antisera are designed α-human (anti-human), α-bovine (anti-bovine), and α-mouse (anti-mouse). The latter was used as a negative control reagent (from [32]).

examined a large number of human × Chinese hamster hybrid cells using human leukocyte antigen (HLA) typing antisera. The parental human cells exhibited reactivity against HLA typing serum 3 only. Most of the hybrids evaluated retained reactivity against this typing serum, and a subset of these displayed reactivity against one or more additional typing sera (i.e., sera 1, 2, 9, and 10) for which the human parental cell was negative.

Immunostaining for HLA antigens may be used for detecting hybrids of parental human cells with differing HLA types, therefore conferring limited utility of this approach for detecting intra-species (human × human) cell hybrids [44, 45]. The results of HLA typing of a human × human hybridoma cell line [46] are displayed in **Table 2**.

As with isoenzyme analysis, immunostaining for surface antigens should be performed as soon as possible once a hybrid cell is created. The results may not in all cases remain the same throughout management of the cell culture over time. For instance, a loss of one or more of the parental surface antigen reactivities may coincide with loss of chromosomal material, and perhaps function, with time in culture.

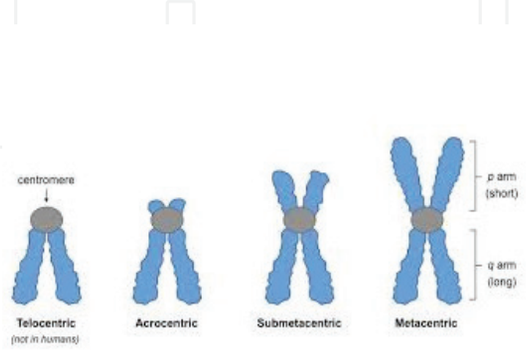
Parameter	Parental cell lines		Hybrid cell line
	GM15006TGOB	EC_{EBV}	GMEC-101
Chromosomes (mean ± standard deviation)	38 ± 3	38 ± 2	84 ± 7
HLA type	A2	A1,2	A1,2
	B18	B5,17	B17
	C7	C6,7	C7
	Bw6	Bw4	Bw4,6
	DR5	DR6	DR5,6

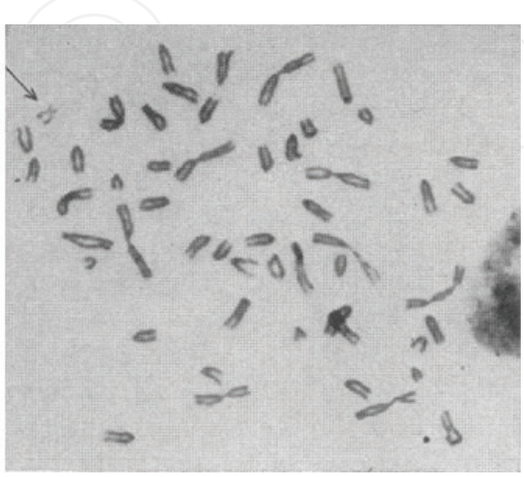
Table 2. Authentication of a human × human hybridoma cell line using HLA typing (data from [46]).

2.3. Karyotypic (cytogenetic) analysis

Karyotypic analysis of inter-species hybrid cells enables an investigator to visualize the rearrangement and addition/deletion of chromosomes that are typically (but not always) observed in such hybrids as a result of the fusion of the two parental cells (**Tables 1** and **2**). The analysis may amount to determination of modal chromosome number and/or the range of chromosomal numbers observed in a set of metaphase spreads [38, 45]. The chromosomes comprising the karyotype may also be analyzed for morphology (telocentricity, acrocentricity, and banding pattern analysis, see **Scheme 1**), enabling assignment of chromosomes to one parental species or the other [26, 32, 36, 46, 47].

Jacobsen and co-workers [22] used fluorescence in situ hybridization (FISH) to demonstrate inter-species cell hybrids between human breast cancer and mouse stromal cells in patient-derived xenografts. The authors labeled human and mouse Cot-1 DNA (enriched in repetitive DNA sequences) with different fluorophores and used these as FISH probes. They were able to highlight the origin of individual nuclei in formalin-fixed, paraffin-embedded tissue





Scheme 1. Part 1. Schema for chromosomal structure. Part 2. Human × mouse karyotype from [36]. The arrow indicates the single human submetacentric chromosome among numerous telocentric mouse chromosomes.

sections, and the origin of individual chromosomes in metaphase spreads, and were able to detect hybrid chromosomes consisting of both human and mouse DNA.

At the time of creation, hybrid cells contain a complement of chromosomes, a portion of which are attributable to one of either parental cells, while some may be of unknown origin (**Table 1**). Inter-species chromosomal rearrangements may also occur in somatic cell hybrids [26]. In many, but not all cases, loss of chromosomes attributed to one or the other parental cell is experienced as the hybrid cells are cultured [26, 31, 32, 35, 36, 46].

2.4. DNA content

Measurement of total or nuclear DNA content is used in characterization of somatic cell hybrids, but is not necessarily intended as an identity test for authenticating such cells. The excess number of chromosomes present in certain cell hybrids relative to the parental cells (discussed above) is also reflected in an increase in DNA content in the hybrid cells. This increase may be detected using microspectrophotometric analysis for nuclear DNA [38] or flow cytometric analysis of propidium iodide-stained cells for total DNA [32]. For instance, while authenticating a series of human × bovine hybrid cell lines, van Olphen and Mittal [32] found that the total DNA content of the hybrid cells was 51–77% greater than the average value for the parental cells (**Table 1**).

Levels of nuclear DNA in bovine × mouse hybrid cells corresponded to the relative increases in chromosome count for the hybrids [38]. A hybrid with mean chromosomal count of 53 (vs. parental values of 44 and 44) was found to have nuclear DNA content similar to the parental mouse cell, while a hybrid with mean chromosome count of 89 displayed a bimodal nuclear DNA content, with one peak similar to that of the parental mouse cell and another peak at around twice the parental cell peak value [38].

Both total DNA and chromosome count for a hybrid cell may evolve with continued passage of a culture, due to the propensity for loss of chromosomes derived from one or both parental cells, as mentioned above.

2.5. Nucleic acid sequence-based methods

The ability to detect intra-species and inter-species cell hybrids has been greatly facilitated by the development of nucleic acid sequencing methods, such as DNA barcoding (PCR- or sequence-based approaches targeting mitochondrial genes), STR analysis, and next generation sequencing.

2.5.1. DNA barcoding

Ono et al. [48] used a nested PCR targeting the cytochrome b gene of 7 animal species (human, mouse, rat, rabbit, cat, cow, and pig) to authenticate two inter-species hybrid cell lines. These included the 4G12 hybridoma cell line (human B lymphocyte × mouse myeloma) and the N18-RE-105 hybridoma cell line (mouse glioma × rat neural retina). Cytoplasmic isoenzymes from the two hybridoma cell lines were found to display human- or rat-specific migration patterns in isoenzyme analysis, but yielded a result expected for mouse in the nested PCR (**Figure 3**). The authors concluded that the preferential retention of mouse mitochondria in

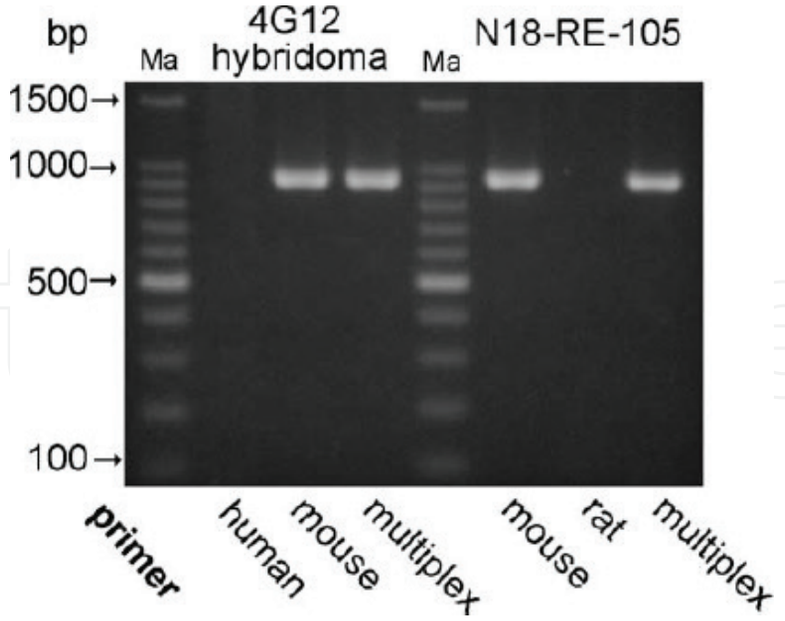


Figure 3. Use of nested PCR to evaluate a human × mouse hybrid cell line [4G12] and a mouse × rat hybrid cell line [N18-RE-105]. Multiplex group 1 (targeting human, mouse, rat, rabbit, cat, cow, and pig) or the corresponding species-specific primer pairs were used. Note that only the mouse DNA is detected in these two hybrid cells (from [48]).

hybrid cells in which one of the parental cells was mouse, as observed also by Attardi and Attardi [47], diminishes the utility of the nested PCR method for identifying inter-species cell hybrids.

2.5.2. STR profiling

Chan et al. [49] used STR profiling to identify presumed intra-species human hybrid cell lines comprised of HeLa × EBV-negative NPC (Epstein-Barr virus-negative nasopharyngeal carcinoma) cells. Four EBV-negative NPC cell lines (CNE-1, CNE-2, HNE-1, and HNE-2) were found to have STR profiles similar to each other, and also shared at least one allele with HeLa across 16 STR loci, as well as additional alleles at several of the STR loci that were attributed to an unknown EBV-negative NPC cell. High-throughput RNA sequencing by Strong et al. [50] resulted in similar conclusions and raised similar concerns for three other EBV-negative NPC cell lines (HONE-1, AdAH, and NPC-KT).

Because these particular EBV-negative NPC cell lines have additional alleles, they do not satisfy the usual match criteria for human cell line authentication [51]. However, it is important to consider all available evidence when deciding if cross-contamination has occurred. The data from Chan et al. [49] showed that CNE-1, CNE-2, HNE-1, and HNE-2 carry an allelic variant (D13S317 13.3) that is characteristic of HeLa derivatives [51]. Strong et al. [50] showed that CNE-1, CNE-2, HONE-1, AdAH, and NPC-KT carry human papillomavirus 18 (HPV18), which is an unexpected finding for NPC cell lines, and display viral and cellular genomic rearrangements that are consistent with HeLa. Looking at all the evidence, it is reasonable to conclude that these EBV-negative NPC cell lines represent not simply cross-contamination with HeLa, but rather somatic cell hybridization with HeLa.

The mechanism responsible for somatic cell hybridization in these seven EBV-negative NPC cell lines is not known. Cell-cell fusion may have occurred between HeLa and an unknown NPC cell line through exposure to Sendai virus. NPC-KT, one of the cell lines investigated by Strong et al. [50], was established by using Sendai virus to fuse AdAH and primary NPC cells [52]. NPC-KT also carried EBV, which can cause cell–cell fusion in monolayer cultures [5, 53]. If the originating laboratory unknowingly performed this work on a culture that was cross-contaminated with HeLa, it may have resulted in a HeLa fusion cell line, which may have subsequently cross-contaminated other cell lines used by the NPC research community. HeLa cells also contain the gene for HPV18 viral protein E5, which is fusogenic [54]. The E5 protein of HPV16 is a fusogenic membrane protein and if expressed in two cells, the cells can fuse [55, 56]. So if HeLa and another cell line expressed the HPV18 analogue protein E5, this could have also induced fusion.

Yoshino et al. [33] used an STR profiling approach to authenticate a series of mouse cell lines, including an inter-strain hybrid (Balb/c mouse × C3H/He mouse) cell line. In this approach, F1 hybrid cells derived from the two parental mouse strains displayed different alleles at each locus, corresponding to the alleles contributed by the two parental strains. Loss of heterozygosity occurring during extended culture was thought to result in loss of one of the parental alleles at the D5 Mit201.1 locus of the dinucleotide STR marker (**Table 3**).

Almeida et al. [34] described results obtained during authentication of an intra-strain (Balb/c mouse) hybridoma (P3X63Ag8.653 × Balb/c mouse splenic cell). In this case, not unexpectedly, identical alleles were detected for eight of nine STR loci evaluated. At the mouse STR 9-2 locus, the parental P3X63Ag8.653 cell was heterozygous, displaying alleles with 15 and 16 repeats, while the hybrid cell contained only the allele with 15 repeats.

Authentication of hybridoma cell lines is difficult, because of the inbred rodent populations that are used for hybridoma generation. Koren et al. [57] proposed a unique solution using degenerate primers to amplify and sequence the variable regions of the monoclonal antibodies produced by their hybridoma cell lines. Because these regions are highly diverse, they can potentially be used to uniquely identify the hybridoma cell line from which a monoclonal antibody is generated. Koren et al. [57] used this approach to resolve a misidentified cell line in their own laboratory, but the method would be useful for any laboratory working with hybridoma cell lines.

Strain/cell line	Size of amplicons for alleles at STR loci					
	D1 Mit159.1	**D2 Mit395.1**	**D4 Mit170.1**	**D5 Mit201.1**	**D13 Mit256.1**	**D17 Mit51.1**
Parental Balb/c	141.8	135.5	242.5	94.9	88.3	155.1
Parental C3H/He	185.1	123.8	236.4	92.5	78.4	140.1
Hybrid UV.CC3.11.1	142.0, 185.2	135.5, 123.9	242.6, 236.4	94.8	88.4, 78.5	155.2, 140.2

Table 3. Authentication of an inter-strain hybrid (mouse) cell line by STR profiling (data from [33]).

2.5.3. Next generation sequencing

Inter-species and intra-species cell fusion may be detectable by next generation sequencing techniques because of the extensive amount of DNA sequencing and the unbiased selection of the DNA segments (i.e., not using species-specific primers for PCR or selection of DNA fragments). A difficulty may occur in genomic regions that are highly conserved between species for which only small sequence differences exist (e.g., a single or a few bases). In such cases, it may be difficult to determine whether the observed difference is a single nucleotide variation (SNV) between two species or between two samples from the same species. Also, SNPs/SNVs are generally transitional sequence changes (i.e., either purine to purine or pyrimidine to pyrimidine) and may not provide sufficient information to determine whether a sample contains cells from two different species or from two different individuals of the same species. To overcome this difficulty, one must sequence DNA segments that are highly variable and unique to different individuals. These might include human, mouse [34], or rat [58, 59] STR and human [60, 61] or mouse SNP arrays [62, 63]. Multiple genomic regions must be evaluated for those cases in which a few or even a single chromosome from one species is retained by the hybrid cell, as is the case with many hybridoma cell lines.

3. Discussion

Hybrid cell lines represent a special problem for the various approaches that have been utilized for authentication up to now. Firstly, the endpoints that are used in cell authentication assays are ultimately, if not directly, dependent upon the genetic make-up of the cell. Intra-species and inter-species hybrid cells are difficult to test because they contain an assortment of genetic material conferred from the two parental cell types during the fusion process. Thus, specific isoforms of enzymes, the presence or absence of surface antigens, chromosome count, and total DNA content are each subject to the assortment of genetic material that is present in the hybrid cell line following the fusion process. This difficulty also applies to the molecular-based methods that are so useful for determining the authenticity of cells. Thus, one cannot predict, in advance, the results that will be obtained during authentication of a hybrid cell using one of these analytical techniques.

Secondly, not all of the genetic material in the hybrid cell is stable, as it is not uncommon for one or more chromosomes to be lost from hybrid cells on continued passage of the culture. This means that the authentication profile of a hybrid cell may evolve over time in culture.

Due to these considerations, a hybrid cell should be evaluated as soon as possible after fusion to get a baseline (reference) profile. Any drift or change in subsequent profiles may imply changes within the chromosome number or expression profiles. The profile of authentication resulting from one or more of these methods can then be used as a sort of fingerprint or reference against which subsequent authentication can be compared (as in authentication of a master cell bank or a working cell bank). Evolution of such a reference pattern may occur as chromosomes are lost, sometimes quite soon following fusion, from a hybrid during continued culture. This evolution typically involves the loss of characteristics of one of the parental cells

(be it parental chromosome(s), species-specific isoenzyme bands or hybrid bands, loss of heterozygosity in SNP or STR profiles, loss of surface staining characteristic of one of the parental cells, etc.). On the other hand, gains in chromosome counts, addition of new isoenzyme bands, appearance of new alleles in SNP or STR profiles, or increases in DNA content with time in culture would not be expected, and such would be considered a red flag during authentication. For instance, such a result might indicate the presence of a cross-contaminating cell type.

4. Executive summary

- Fusion of two different cell types to create an inter-species or intra-species hybrid results in an unpredictable assortment of genetic material derived from one or the other parental cell into the hybrid.
- The outcome of the fusion process in terms of genetic content contributed by the two parental cells will impact the results of the methodologies typically used for determining cell line authenticity.
- Isoenzyme analysis may indicate the presence of electrophoretic bands migrating as expected for one parental cell, the other parental cell, or bands migrating differently than expected for either parental cell.
- Hybrid cells may retain surface antigens characteristic of one parental cell or the other, or of both.
- Chromosome number (total, and number derived from one parental cell or the other) and total DNA content will vary from hybrid to hybrid.
- Human chromosomes found in inter-species hybrid cells tend to be unstable, and are often lost over time in culture.
- Regardless of the authentication method to be used, it is recommended that a baseline evaluation be performed as soon as possible after the fusion process used to create the hybrid cell, and that the result be used as a reference against which future authentication results may be compared.

5. Future perspectives

Hybrid cell lines require new methods to ensure that cell-cell fusion is detected and such cultures can be authenticated to demonstrate their validity as research models. Detection of spontaneous cell-cell fusion is particularly important. Somatic cell hybridization may arise when using feeder layers in vitro or when working with patient-derived xenograft models in vivo. In many cases, cell–cell fusion is associated with increased tumorigenicity or the development of malignant behavior in adjacent cell populations [21, 22]. This has the potential to alter the behavior of patient-derived xenograft models, which may be used as the final step before a novel drug proceeds to clinical evaluation [64].

Current authentication methods are not always effective to detect hybrid cell lines. The advantages and limitations of the available methods are displayed in **Table 4**. Common to all methods is the fact that the chromosomal contributions from each parental cell are not possible to predict in advance. This means that chromosomal makeup and number and corresponding genetic information (e.g., surface antigens, HLA types, enzyme isoforms, alleles at STR loci) will be unique to each fusion cell. In hybrid cells, STR profiles become more

Method	Principle	Advantages	Disadvantages
Isoenzyme analysis	Electrophoretic mobilities of cytosolic enzymes in the fusion cell correspond to one or both parental cell isoforms	Rapid; inexpensive; visual endpoint; useful for inter-species hybrids	Reagents no longer commercially available; results must be compared to a reference result; certain isoforms may be lost with continued passage of the hybrid cells
Immunostaining	Surface antigens of one or both parental cells may be retained	Rapid, visual endpoint; useful for inter-species hybrids; HLA immunostaining enables detection of intra-species (human) fusion cells	Requires species- or HLA type-specific immunostaining reagents and fluorescent microscopy; results must be compared to a reference result; certain surface antigens may be lost with continued passage of the hybrid cells
Karyotyping (cytogenetic analysis)	Chromosomes contributed by two parental cells may be directly observed in the fusion cell	Visual endpoint; useful for inter-species hybrids; can directly determine chromosomal makeup derived from the parental cells; useful for inter-species hybrids	Preparing and interpreting karyotypes takes expertise; human chromosomes in inter-species hybrids are often lost with passage in culture
DNA content	Fusion cells may contain more total DNA content than either parental cell	Total DNA content is simple to measure; DNA content is useful in detecting intra- as well as inter-species hybrid cells	Total DNA content in hybrid cells is often not stable with passage in culture as chromosomes may be lost
DNA barcoding	Mitochondrial DNA sequences are conserved within species	Rapid; DNA barcoding is used for species of origin confirmation of cells	Nested PCR must be created with species-specific primers for parental cells; preferential retention of mouse mitochondria in inter-species bybrid cells diminishes utility of this method
STR profiling	Multiple (8 or more) STR loci provide identity to the donor level	Rapid; STR profiling can enable authentication of human, dog, or mouse cells to the individual donor level; useful for authenticating mouse hybridoma cells	Not typically useful for inter-species hybrid cells; loss of heterozygosity during extended passage in culture may result in loss of STR alleles
Next generation sequencing	Agnostic sequencing of variable DNA sequences such as STR or SNP arrays	Detects inter- and intra-species hybrid cells	Multiple genomic regions must be evaluated in hybrids containing few or single chromosomes from one of the parental cells

Table 4. Limitations and advantages of methods for authenticating hybrid cells.

complex and difficult to interpret, while mitochondrial-based methods may not be effective for species detection if mitochondria from one species are retained preferentially (as is true for the mouse). However, many methods can be optimized to allow for detection of cell-cell hybrids. For example, SNP genotyping is increasingly used for cell line authentication and has been used as a test method for patient-derived xenograft models [64]. SNP panels could be modified to include species-specific marker sets, or human and mouse SNP panels could be run in parallel to confirm species and strain identifications and search for additional markers. Although this type of comprehensive assessment is not usually performed, it could be incorporated into testing pipelines if laboratories are aware of the spontaneous (unintentional) cell fusion issue and look specifically for such markers of cell fusion. A role has been suggested for cell-cell fusion in cancer, stem cell plasticity, and trans-differentiation [17–20, 54, 59, 65–67]. A better set of tools is needed to explore hybrid cell lines and the role of somatic cell hybridization in health and disease.

Abbreviations

DNA	deoxyribonucleic acid
EBV	Epstein-Barr virus
FISH	fluorescence in situ hybridization
HHV-8/KSHV	herpesviruses-8/Kaposi sarcoma herpesvirus
HLA	human leukocyte antigen
HPV	human papillomavirus
HTLV-1	human T-cell lymphotropic virus type 1
NPC	nasopharyngeal carcinoma
PCR	polymerase chain reaction
RFLP	restriction length fragment polymorphism
RNA	ribonucleic acid
SNP	single nucleotide polymorphism
SNV	single nucleotide variation
STR	short tandem repeat
SV40	simian virus 40

Author details

Raymond W. Nims[1]*, Amanda Capes-Davis[2], Christopher Korch[3] and Yvonne A. Reid[4]

*Address all correspondence to: rnims@rmcpharma.com

1 RMC Pharmaceutical Solutions, Inc., Longmont, CO, USA

2 CellBank Australia, Children's Medical Research Institute, The University of Sydney, Westmead, New South Wales, Australia

3 Division of Medical Oncology, University of Colorado Denver, Aurora, CO, USA

4 ATCC, Manassas, VA, USA

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