Review

Chicken Cells – Oncogene Transformation, Immortalization and More

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Abstract. Domestic chicken as a laboratory animal as well as chicken cells *in vitro* have been highly evaluated in several fields of experimental biology. Retrovirology and experimental oncology traditionally use this model, whose comparative aspects are still inspirative. The first (retro)viral aetiology of a tumour was recognized in the chicken and the first quantitative *in vitro* measurement of oncogenic transformation was developed using the chicken cells. Chicken cells (like human and primate, but unlike rodent cells) have a long primary life-span, during which they remain genetically stable. While this property is advantageous for several types of experiments, it correlates with a low propensity of the chicken cells to immortalization. Recent establishment of several continuous chicken cell lines, however, has surmounted this drawback. Furthermore, the chicken B cell line DT40 was proved to be extremely useful for gene disruption studies because of a high frequency of gene targeting not found in any vertebrate cells. In the present communication, we have tried to review several traditional achievements accomplished using the chicken model and point to newly opened areas, where chicken cells appear to be an efficient tool, particularly in cell transformation and immortalization.

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Abbreviations: AEV – avian erythroblastosis virus, AID – activation-induced deaminase, ALV – avian leukosis virus, AMV – avian myeloblastosis virus, CEF – chicken embryo fibroblast, cMGF – chicken myelomonocytic growth factor, EGFR – epidermal growth factor receptor, HDF – human diploid fibroblast, LTR – long terminal repeat, MHC – major histocompatibility complex, PI3-K – phosphatidylinositol 3 kinase, PTK – protein tyrosine kinase, REF – rat embryo fibroblast, RSV – Rous sarcoma virus, SCF – stem cell factor, *ts* – temperature sensitive, TGFα – transforming growth factorα.

Rous sarcoma virus (RSV) and its oncogene v-*src* – the longest studied retrovirus and a prototype oncogene

RSV is not only the oldest known tumour (retro)virus (Rous, 1965), but together with other avian retroviruses it has played an important role in fundamental achievements in the development of the whole field of retrovirology. Among others we should mention the focus assay, the first quantitative *in vitro* measurement of oncogenic transformation, the discovery of reverse transcriptase, the identification of the first oncogene v-*src* and its cellular origin, the discovery of temperature sensitive (*ts*) mutants affecting transformation by oncogenes, etc. (Temin and Rubin, 1958; Baltimore, 1970; Temin and Mizutani, 1970; Wyke, 1973; Stehelin et al., 1976; Brugge and Erickson, 1977).

RSV, like other transforming retroviruses, acquired the coding regions of the normal cell c-*onc* gene, in this case c-*src*, a prototype of the Src-family of protein tyrosine kinases (PTKs). In the course of recombination with a retrovirus, c-*src* has been truncated at the carboxy end of the last coding exon and several amino acids of the host genome origin have been substituted. Of key importance is loss of tyrosine 527, the main autoregulatory phosphorylated residue that allows for constitutive PTK activation (reviewed in Shalloway and Taylor, 1997; Thomas and Brugge, 1997).

One drawback of avian retrovirology (as well as of immunology, etc.) is the paucity of continuous cell lines. This is caused by a very low propensity of the chicken cells, like primate and human cells, to immortalization (Hayflick, 1965; Beug and Graf, 1977). On the other hand, some experimental models can take advantage of the long primary *in vitro* life-span of the chicken cells, or of rare cases of immortalization (Table 1).

Epigenetic silencing of integrated RSV

RSV is an example of a retrovirus which is strongly specialized to its original host and can productively infect only chickens and several closely related avian species. When transmitted into rodent cells, it does not propagate because of multiple blocks at all levels of its replication cycle. In contrast to chicken embryo fibro-

Table 1. A survey of some experimental models using the chicken cells

Cell type	Characteristics	Experimental exploitation	Citations
Non-transformed immortal CEF	Spontaneously immortalized clones and cell lines (DF-1) from senescent cells in culture Derived from ev-0 chickens Infinite growth > 200 subcultures	Molecular biology studies of avian retroviruses, their oncogenes and process of immortalization	Himly et al., 1998 Schaefer-Klein et al., 1998, and elsewhere - see the text
Normal erythroid progenitor cells	Chicken erythrocytic progenitors are endowed with high "self renewing" potential (> 25 cell divisions before commitment to differentiation)	Biochemical investigations of response to differentiation signals	Hayman et al., 1993 Müllner et al., 1996 (review)
Transformed haematopoietic cells	Specific subsets of haematopoietic cells selectively transformed by different oncogenes of acute ALV Several cell lines. Conditional ts mutant oncogenes available	Molecular biology studies on the mechanism of haematopoietic cell differentiation and oncogenic transformation	Beug et al., 1979, 1981, 1982, Coll et al., 1983 Moscovici and Gazzolo, 1982, Graf et al., 1992 McNagny and Graf, 1996 (review)
v-*src* DNA-transformed sarcoma cells	Several transplantable tumours and tumour cell lines. Derived from tumours induced by and maintaining the LTR,*v-src*, LTR provirus in *MHC(B)* congenic chicken lines. Infinite growth > 200 subcultures	Studies of the v-Src function in initiation and maintenance of the transformed phenotype, in vivo oncogenicity and cell immortalization	Svoboda et al., 1992 Plachý et al., 1994 Plachý, 2000 (review) Svoboda, 2000 (review)
ALV-induced B-cell tumour	DT40 cell line and its variants High rate of targeted integration, continually diversify Ig by gene conversion	Molecular mechanisms of homologous recombination Genome mapping	Bezzubova et al., 1997 Arakawa et al., 2002

blasts (CEF), the non-permissive mammalian cells lack proper receptor molecules on the cell surface, integrated RSV proviruses are poorly expressed, the ratio of spliced and genomic retroviral mRNAs is aberrant, and virions do not assemble because of defective polyprotein cleavage and absence of certain cellular factor(s) necessary for virus budding. Whereas most of these blocks represent merely peculiar interactions between the virus and the non-permissive cell, the inefficient transcription probably reflects more general differences between chicken and mammalian cells. Transcriptional suppression is clearly a post-integration event related to the epigenetic state of RSV long terminal repeats (LTR) as shown by efficient transient LTR-driven expression of v-*src* or reporter genes after transfection into mammalian cells (Overbeek et al., 1986; Hejnar et al., 1999) and by untackled transforming potential of molecularly cloned silenced RSV proviruses (Hejnar et al., 1994). LTRs of RSV proviruses integrated in mammalian cells were found to be heavily methylated (Katz et al., 1983; Searle et al., 1984; Hejnar et al., 1994) whereas unmethylated in chicken cells (Katz et al., 1983; Blažková et al., unpublished). The importance of DNA methylation for the silencing of RSV was documented by the protection of RSV-based reporters from silencing by an anti-methylation tag, the CpG island of mouse adenosine-phosphoribosyltransferase gene (Hejnar et al., 2001). Furthermore, differences in the transient expression level after transfection of *in vitro* methylated reporters into chicken and mammalian cells show different sensitivity of RSV LTRs in these cells (Hejnar et al., 1999).

Taken together, these results point to differences in the global DNA methylation machinery and the capacity for methylation-dependent transcriptional repression between avian and mammalian cells. Such differences should be expected, because birds and mammals arose independently from their reptilian ancestors where, according to the genomic methylation level and CpG island differentiation (Jabbari et al., 1997), the control of transcription by DNA methylation is much less important than in warm-blooded vertebrates.

Permissiveness of avian *vs* mammalian cells to v-*src* oncogenesis

The v-*src* oncogene, as a transforming counterpart of the chicken c-*src* gene, readily transforms CEFs *in vitro* and induces sarcomas in chicks and in related avian

species *in vivo*. There is also a broad scale of mammalian species whose embryonic cells are sensitive to the v-*src* transformation. These include rodents (mouse, rat, field vole, Syrian hamster), dogs, and kangaroos, but not humans, apes and monkeys. Even the mouse and rat cells differ significantly in their propensity to be transformed by v-*src* oncogenes transduced by various RSV strains. For example, mouse NIH 3T3 cells resist to the transformation by v-*src* variants mutated outside the catalytic domain of the v-Src kinase (Reddy et al., 1988) and Swiss 3T3 cells, which do not respond mitotically to phorbol-ester, are completely refractory to the v-*src* effects (Nori et al. 1990). Even more, Inoue et al. (1995) described that rat embryo fibroblasts (REF) can transduce this refractoriness into other cells in cell fusion experiments, suggesting a suppressive factor(s) expressed in REFs. In another study, Hjelle et al. (1988) demonstrated the efficient transformation of REFs but not of human diploid fibroblasts (HDF) by v-*src*. Keeping in mind the truncation of the human c-*src* gene implicated in the progression of colorectal carcinogenesis (Irby et al., 1999), it is curious that the v-*src* oncogene is ineffective in transformation of human cells. The only cell type successfully transformed by v-*src* so far are the proximal tubule kidney cells (Nanus et al. 1991), the target of renal cell carcinogenesis. Critical evaluation of the aforementioned results, comparison of structural features and substrate specificities of both chicken and human *src* gene products, as well as construction of recombinant chicken-human *src* chimeras could be of tremendous importance in understanding the species- and cell type-dependent sensitivity/resistance to the transformation by activated *src*. Regardless that the aforementioned studies are rather old and sometimes controversial, comparison of chicken and human fibroblasts – two extremes as concerns the sensitivity to v-*src* transformation – might have a say to the downstream requirements of v-*src* tyrosine kinase in the process of cell transformation and to the tumorigenic potential of the human c-*src* gene (see Irby and Yeatman (2000), Frame (2002) for review on Src in human cancer).

Temperature sensitivity of v-*onc* genes

Because avian cells tolerate larger temperature shifts than their mammalian counterparts, it has been relatively easy to select for temperature sensitive mutations in transforming proteins and address their function by inactivating and reactivating them at will. Fibroblasts, primary cells of mesenchymal origin, become quiescent when deprived of growth factors (serum) or matrix interaction. Such quiescent cells nevertheless remain viable for some time. In contrast, the same cells transformed by RSV do not enter viable quiescence, they adhere loosely to the substratum, and they either multiply in suspension or die. This phenomenon was originally also used for selection of *ts* transformation mutants of RSV (Wyke, 1973).

Comparative cell culture models of *ts* v-*src*-expressing chick embryo fibroblasts (CEF) and rat embryo fibroblasts (REF) were used in studies of the Src-dependent focal adhesion assembly and turnover. The spatial and temporal regulation of the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) upon these processes was also studied (Fincham et al., 1996, 2000; Fincham and Frame, 1998; Frame et al., 2002). The temperature sensitivity of v-*src* was explored for characterization of the mitogenic effects of Src (Wyke et al., 1993, 1995; Johnson et al., 1998) and its role in apoptosis (Johnson et al., 2000). The role of v-Src in apoptosis seems to be ambiguous, because it can both prime for and protect against apoptosis depending on the presence of other signals like serum growth factors and the cellular model used (*ts*-v-*src*-transformed Rat-1 cells; v-*src*-transformed CEFs; mouse cell line NIH3T3). A clear antiapoptotic effect was seen in transformed *ts LA29* Rat-1 cells grown under low serum conditions. In this experiment, model cells continued to cycle at the permissive temperature (v-*src* switched on) and were rapidly committed to apoptosis after a shift to the non-permissive temperature (v-*src* switched off). The antiapoptotic effect of v-Src was mediated apparently through direct activation of phosphatidylinositol 3-kinase (PI3-K) and hence its signalling pathway leading to serine/threonine kinase c-Akt (Johnson et al., 2000; for review about Akt see Downward, 1998). The role of the PI3-kinase pathway and Akt in protection of v-*src*-transformed cells from experimentally induced apoptosis was also suggested by Hakak et al. (2000). Another effect of v-*src*, activation of the *bcl-2* antiapoptotic pathway, was reported using chicken RSV-transformed cells (Gillet et al., 1995).

The development of *ts* mutants of viral oncogenes of acute avian leukosis viruses (ALVs) – v-*erbB*, v-*myb*, v-*ets*, v-*rel* (Graf and Beug, 1978; Beug et al., 1982; Golay et al., 1988; Gilmore, 1991; Graf et al., 1992) – provided a genetic approach to the study of haematopoiesis, consisting of the expansion of oncogene-transformed cells at the permissive temperature and subsequent inactivation of the oncoprotein by shifting these cells to the non-permissive temperature (see also below).

Transformation, oncogenesis and immortalization of (tumour) cells

Replicative senescence is known to be an intrinsic mechanism in determining the finite life-span of *in vitro* cultured cells. While some aspects of replicative senescence are well conserved from yeast to mammalian cells, interesting differences exist even within cells of vertebrate species like human, mouse and chicken. Normal human diploid fibroblasts (HDF) and chicken embryonic fibroblasts (CEF) were shown to have a more extended *in vitro* life-span (30 to 50 population doublings in cell culture) than their mouse embryonic