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Insights into the Role of Genetic Alterations in Adrenocortical Tumorigenesis

M. Herbet[a,b], J.J. Feige[a,b] and M. Thomas[a,b,*]

[a] Institut National de la Santé et de la Recherche Médicale, Unité 878, Grenoble, France
[b] Commissariat à l'Energie Atomique, Institut de Recherches en Technologies et Sciences pour le Vivant, Grenoble, France

[*]Corresponding author at: INSERM, U878, iRTSV-LAPV, CEA-G, 17 rue des Martyrs, 38054 Grenoble, Cedex 09, France. Tel.: +33 1 438 78 44 64; fax: +33 1 438 78 50 58.
E-mail address: michael.thomas@cea.fr (M. Thomas)

Abstract

Whereas benign adrenocortical tumors are frequent in the population, adrenocortical carcinoma (ACC) is a rare cancer. Significant advances in the understanding of the pathogenesis of sporadic ACCs have been possible through the study of hereditary syndromes responsible for ACCs. The genetic alterations involved in these syndromes have also been found in sporadic ACCs. Several specific genes have been shown to be altered in sporadic ACCs. Despite these progresses, the underlying sequence(s) of events remains( ) to be elucidated. Progressive transformation of a normal tissue into a benign tumor and ultimately into a carcinoma occurs via accumulation of genetic and epigenetic alterations. Likewise, a multistage model has been proposed for the adrenal tumor development. This review summarizes the molecular alterations likely involved in the multistage tumorigenesis and describes a mouse model which allows us to evaluate the effect of individual genes or combination of genes in the development of adrenocortical tumors.

Keywords:
Adrenal cortex; Adenomas; Carcinomas; Multistage tumorigenesis; Xenotransplantation

1. Introduction

Cancer is a disorder at the cellular DNA level owing to the accumulation of multiple genetic changes resulting in the dysregulation/failure of genes that control cell cycle and cell proliferation. Some of the observed genetic alterations are widely shared among the different tumor types; however, the genetic analysis of cancers reveals a number of defined mutational events that appear specific for particular cancers, indicating that cancers follow certain evolutionary paths. Therefore, the comprehensive knowledge of a broad field of genetic alterations in a tumor type and the study of the correlation between these alterations and the resultant phenotype allow to better define the tumor classification and the understanding of the multistage carcinogenesis process. Finally, the comprehension of the signaling mechanisms and pathways that underlie the pathogenesis of cancer is critical to the development of more effective detection and therapeutic strategies.

The ability to recapitulate human cancer pathogenesis in a mouse model represents an important part of cancer research. The availability of athymic mice (*nu/nu*) and subsequent immunodeficient mouse strains with other genetic lesions such as severe combined immunodeficiency (scid) allowed the widespread possibility of studying human tumor explants and cell lines grown as xenotransplants. Xenografts are derived either from patient biopsies or from continuous cell lines. Biopsies have the advantage to retain the morphological and molecular marker properties reminiscent of the original tumors in humans. However, determining which specific oncogene and/or tumor suppressor gene mutations co-operate in tumor initiation and progression is an almost impossible challenge to take up. In contrast, cell lines from human tumors generally show a more homogeneous, undifferentiated histology indicative of a higher selection pressure *in vitro* during long-term growth in culture. Moreover, one needs to bear in mind that these cell lines have an undefined and complex mutational history from which it is often difficult to decipher the molecular events that lead to their creation and possibly, although not always, without resemblance to the human disease histology and architecture. Another important variable, which has to be considered when interpreting xenograft studies, is the site of tumor implantation. Most xenograft experiments use ectopic sites such as subcutaneous implantation where it is easy to inject tumor cells from culture or to transplant a small tumor mass and to assess tumor growth through the use of calipers to determine tumor volume. Several reports have shown differences in biological behavior such as ability to metastasize, response to antitumoral therapy when tumors are grown subcutaneously relative to orthotopically (Eccles et al., 1994).

Genetically engineered mouse models have been invaluable for the study of human cancer. Indeed, mice are inexpensive to keep, their generation is short, and they have in general large litters. The various mouse strains are highly inbred, providing uniform conditions in which experiments can be easily reproduced and statistical significance achieved. Currently, mouse modeling of human cancer is possible through the expression of oncogenes, specific genetic mutations, or the inactivation of tumor suppressor genes. However, mouse models may fail to faithfully mirror the human disease because the spontaneous occurrence of carcinoma in mice is rare. As they age, most laboratory mice will develop sarcomas and lymphomas while aged humans will develop carcinomas originating from the epithelial cells of various tissues (DePinho, 2000). Moreover, another fundamental difference between cancer development in humans and in transgenic mice is that, the genetic alteration, i.e. gain- or loss-of-function of a gene typically occurs in either all cells of the mouse or in all cells of a particular tissue, which contrasts with human cancer in which gene alterations are typically rare and stochastic.

2. Genetic Alterations in Sporadic Adrenocortical Tumors

Sporadic adrenal carcinoma (ACC) is a rare endocrine neoplasm in humans, notorious for its aggressive behavior, metastatic potential and poor outcome with 5-yr survival ranging from 16 to 38 % (Allolio and Fassnacht, 2006; Kirschner, 2006). To date, radical surgery remains the mainstay of the curative modality put forward by clinicians for ACC patients (Kopf et al., 2001; Shen et al., 2005; Allolio and Fassnacht, 2006). Twenty percent of ACC patients with advanced disease cannot be cured by surgery (Crucitti et al., 1996; Icard et al., 2001) and the benefit of the medical treatment with mitotane (o,p'-dichlorodiphenyldichloroethane) is still questionable (Wooten and King, 1993; Latronico and Chrousos, 1997). By contrast, benign adrenocortical adenomas (ACAs) are common in the general population and are generally found incidentally (Grumbach et al., 2003). Whether adenoma represents a separate entity or is in fact part of a process of tumor progression leading to the emergence of an ACC is still an open question. The prevalence rate of cortical adenomas in a series of surgically resected incidental tumors is of 53% (Angeli et al., 1997) whereas the incidence for ACC is between 4 to 12 new cases per million in adults (Grumbach et al., 2003). From those numbers, it is clear that the frequency of adenomas is much higher than the frequency of ACC, which is consistent with the fact that only a very small fraction of adenomas will progress to cancer in patients. The development of tumors in other tissues, such as the colon is based on the accumulation of multiple genetic changes, resulting in progression from benign to malignant diseases. Most adenomatous polyps of the colon, even though they are the precursors of invasive cancer, never actually progress to that stage (Vogelstein and Kinzler, 1993). This is consistent with the general concept of multistage tumorigenesis; the additional genetic change(s) that an adenoma needs to become a carcinoma is (are) infrequent. Thus, clinically, the occurrence of benign tumors is much more frequent than carcinomas.

Progress into the elucidation of the genes and pathways involved in the pathogenesis of sporadic ACC has been slow largely because of the rarity of this tumor. However, the study of two hereditary tumor syndromes associated with adrenal neoplasms has helped to unravel some genetic alterations.

2.1. The Li-Fraumeni syndrome

The patients affected by this autosomal dominant familial cancer syndrome have susceptibility to breast carcinoma, brain tumors, soft tissue sarcomas, leukemia and ACC (Hisada et al., 1998). The underlying genetic alteration is a germline mutation of TP53 located at 17p13. The TP53 gene is a tumor suppressor gene and is the most frequently mutated gene in human cancers (Hollstein et al., 1991). The p53 protein controls the cell cycle at the G1/S interface and plays an important role in inducing programmed cell death in response to severe cellular DNA damage (Vogelstein et al., 2000). Somatic mutations of TP53 are mostly located within exons 5-8 and are found in 20 to 33% of sporadic ACC whereas in sporadic ACAs the frequency rate is between 0 to 6 %, suggesting that genetic alterations in TP53 gene are rather involved late in the process of evolution towards malignancy (Ohgaki et al., 1993; Reincke et al., 1994; Libé et al., 2007). Loss of TP53 has at least three roles in progression: suppressing apoptosis, preventing cell cycle arrest and permitting genetic instability, which may be in favor of the generation of viable genetic variants (Shao et al., 2000).

2.2. The Beckwith-Wiedemann syndrome

This syndrome is an autosomal dominant familial disease characterized in affected patients by macroglosia, exomphalos, gigantism, and development of embryonic tumors such as Wilms' tumor, hepatoblastoma, rhabdomyosarcoma, and ACC (Maher and Reik, 2000) (Hertel et al., 2003). The gene locus responsible for this syndrome was mapped to

chromosome 11p15 (Henry et al., 1989), which includes the Insulin-like Growth Factor 2 (IGF-2), H19, and cyclin-dependent kinase inhibitor C (p57/kip2). This locus is subject to parental imprinting with IGF-2 solely expressed from the paternal allele, and H19 and p57/kip2 normally expressed from the maternal allele. The pathogenesis of the Beckwith-Wiedemann syndrome has been ascribed to genetic and epigenetic changes in the 11p15 locus resulting in overexpression of IGF-2 and low expression of p57/kip2 and H19 (Lam et al., 1999). IGF-2 is predominantly expressed during embryonic development. In the actively growing fetal human adrenal gland, high levels of IGF-2 are detected whereas in adult adrenal tissue, only low IGF-2 levels are found. p57/kip2 is a cyclin-dependent kinase inhibitor and regulates cell cycle progression from the G1 to the S phase. H19 mRNA is not translated to protein and is hypothesized to regulate IGF-2 expression (Maher and Reik, 2000).

Genetic analysis of sporadic adrenocortical tumors for the 11p15 locus have shown that approximatively 90% of ACCs and 8.5% of ACAs overexpressed IGF-2 (Ilvesmaki et al., 1993; Gicquel et al., 1994a, 1997, 2001), suggesting that whether IGF-2 plays a role in the pathogenesis of ACCs, it might be only in late stages of adrenocortical tumor development. Conversely, p57/kip2 and H19 mRNA expression are down-regulated in sporadic ACCs (Bourcigaux et al., 2000; Gicquel et al., 2001).

Several other genetic syndromes such as Carney complex, Mutiple Endocrine Neoplasia type 1 and Mc Cune-Albright syndrome are associated with the development of ACCs. However, the involvement of the specific genetic defect at the origin of these diseases in the pathogenesis of sporadic ACCs has not been clearly established (Schulte et al., 2000; Bertherat et al., 2003) or might play only a minor role in malignant ACC tumor growth.

2.3. Specific genetic alterations in sporadic adrenocortical tumors

The multistage model of tumorigenesis emphasizes somatic mutations as the initiating event leading to the formation of preneoplastic lesion, which then is followed by the accumulation of additional genetic and epigenetic changes in the initiated cells or its progeny. One can hypothesize that preneoplastic lesion could represent an ACA which may become an ACC through the acquisition of new alterations. If the adenoma-to-carcinoma concept is applicable to the adrenal cortex, then common genetic alterations should be found in both ACAs and ACCs as well. Two clinical cases (Bernard et al., 2003; Gaujoux et al., 2008) describing an ACT with a benign and a malignant part are consistent with this concept. The identification of genetic alterations known to be associated with familial cancer syndromes as those discussed above, in sporadic ACCs was of great help in unraveling the genetic lesions involved mostly in progression. Initiation of benign adrenocortical tumors remains a mystery although there are specific genetic alterations occurring in sporadic benign and malignant tumors.

The Ras gene family is composed of three genes (H-, K- and N-Ras) and encodes low molecular weight GTPases which cycle between the GDP-bound (inactive) and GTP-bound (active) state at the plasma membrane. These molecular switches are involved in signaling pathways that modulate proliferation, differentiation, motility and death (Shields et al., 2000). Due to its pivotal roles, it is not surprising that Ras genes are the most frequently mutated oncogenes in human cancer (Bos, 1989). Activating N-Ras mutations were identified in 12.5% of ACCs and ACAs tested whereas no mutations were found in K- and H-Ras (Yashiro et al., 1994). In a smaller number of tumors, Moul *et al.* (1993) did not detect any point mutations in N-, H- or K-Ras. Finally, Ocker *et al.* (2000) also did not identify K-Ras mutations in 40 AAs. It is interesting to note that Epidermal Growth Factor Receptor (EGFR) is overexpressed in ACAs as well as in ACCs (Kamio et al., 1990; Sasano et al., 1994). Moreover, as the signal transducing tyrosine kinase activity of the EGFR is mediated by Ras proteins among others, it is conceivable that chronically active wild type Ras promotes

tumorigenesis through activation of multiple Ras effectors that contribute to deregulated cell growth, dedifferentiation, and increased survival, migration and invasion. EGF is not overexpressed in ACCs, but the receptor may be bound by TGFα, which is a natural ligand for EGFR and is often found in adrenal tumors (Sasano et al., 1994).

Signaling by the Wnt family of secreted lipoproteins has central roles in embryogenesis and in adult tissue homeostatic processes. The central event in the canonical Wnt pathway is the stabilization of the transcription cofactor β-catenin in the cytoplasm and following its nuclear translocation and interaction with T-cell factor/lymphoid enhancer factor, β-catenin-dependent gene expression (Clevers, 2006). β-catenin has also a function in cell-cell adhesion by interacting with E-cadherin and α-catenin. Activating mutations of the Wnt signaling pathway have been described in a large number of sporadic tumors (Giles et al., 2003). Activating mutations of exon 3 of the β-catenin gene (CTNNB1) were found with similar frequencies in ACAs and ACCs whereas abnormal immunolocalization of β-catenin was observed at a higher rate in ACAs than in ACCs (Tissier et al., 2005). This discrepancy could be explained by mutations in other components involved in the Wnt signaling pathway, which may participate in the progression of ACCs towards a more aggressive phenotype. A recent study found similar results as concerns mutation rate in ACAs however, due to the very small number of ACCs included, no mutations in the β-catenin gene were found (Tadjine et al., 2008). The identification of β-catenin mutations in hepatocellular adenomas was correlated to a higher risk of malignant transformation in hepatocellular carcinoma (Zucman-Rossi et al., 2006). Thus, it is possible that β-catenin mutation may be part of the multistage model of tumorigenesis.

Angiogenesis is a pivotal step in the progression of a variety of solid tumors (Folkman, 1992). The angiogenic profile of ACTs may be assessed by the analysis of angiogenic factors such as Vascular Endothelial Growth Factor (VEGF) expression. ACCs appeared to have a higher angiogenic potential as compared to ACAs because of an increase in VEGF expression (de Fraipont et al., 2000; Bernini et al., 2002). This overexpression in ACCs endows the tumor with the capability to synthetize new blood vessels and therefore to induce tumor growth towards malignancy and metastasis. VEGF levels, although difficult to measure in serum due to its abundance in platelets, were reported to be significantly higher in sera of patients with ACCs than of patients with ACAs (Kolomecki et al., 2001).

2.4. Clonal composition of adrenocortical tumors

Specific molecular events underlying the initiation of human adrenocortical tumor formation are poorly understood; however, results of several studies suggest that adenomas and carcinomas arise after somatic mutational events. In particular, clonal composition of ACTs tumors has been determined by the patterns of X-chromosome inactivation in females heterozygous for X-linked polymorphisms. From the three studies carried out thus far, one may conclude that ACCs are more often monoclonal whereas ACAs may be either polyclonal or monoclonal (Beuschlein et al., 1994; Gicquel et al., 1994b; Blanes and Diaz-Cano, 2006). The genetic heterogeneity evidenced in ACAs may be explained either by different pathological mechanisms or, by different stages of a common multistep process. Thus, a somatic mutational event causes one or a small number of adrenocortical cells to initiate the neoplastic process by polyclonal expansion (Nowell, 1976). Subsequent somatic mutations result in additional rounds of clonal expansion towards selection of subclone with an increase in its survival and/or proliferative potential, which will tend to spread in the neoplasm to the detriment of competitor clones and normal cells that lack the beneficial mutation.

3. Contribution of cell transplantation studies to deciphering multistage tumorigenesis in adrenal cortex

Through advances in the molecular analysis of human adrenocortical adenomas and carcinomas as discussed before, several well-defined and sometimes common molecular pathways have been found to be dysregulated. However, the progress into the elucidation of the mechanisms of adrenal tumorigenesis with a stepwise progression from AACs to ACCs has been slow in particular because of the rarity of the ACCs. The lack of a suitable animal model is another obstacle for unraveling the role of a given genetic alteration and its possible cooperation with other gene defect in the pathogenesis of the disease. Recapitulating the various stages of tumor progression of human cells within a mouse may be an important approach to understanding the potential behavior of individual premalignant adrenal lesions and to developing rational medical strategies for their management to halt their progression to invasive cancer.

In order to tackle this issue, we used an *in vivo* model of cell transplantation and tissue reconstruction (Thomas et al., 1997; Thomas and Hornsby, 1999). Since orthotopic adrenal cell implantation in mice is technically very challenging, we have developed a model where normal primary bovine or human adrenocortical cells are transplanted under the kidney capsule of adrenalectomized scid mice (Fig. 1). Once implanted in that space, the cells rapidly reconstitute a vascularized and functional tissue, which secretes cortisol and avoids the otherwise lethal effect of adrenalectomy (Thomas and Hornsby, 1999; Thomas et al., 2002). The tissues formed are chimeric, composed of human or bovine adrenal cells together with mouse cells (endothelial cells lining the capillaries and stromal cells). Tissue reconstruction models differ from conventional assays in immunodeficient mice (subcutaneous or intra-muscular injection of cell suspension) in that the cell survival is not severely compromised by the implantation technique. If the cell survival is low, as it is in conventional assays, an undesired selection advantage might take place among the cells that would lead them to acquire a molecular phenotype different from the one of the general cell population. The fact that clonal bovine adrenocortical cells could form a functional tissue following transplantation (Thomas et al., 1997) prompted us to genetically modify the cells prior transplantation. When genetically modified cells are used during transplantation procedures, they form what may be termed a transgenic tissue (Thomas et al., 2000, 2002; Mazzuco et al., 2006a,b). The power of germline genetic modification in the mouse to answer important biological questions is well established. For human cells, genetic modification in cell culture has been similarly powerful in elucidating human gene function. However, although germ line modification of humans is not an acceptable option, studying transgenic tissues containing human cells within experimental animals is acceptable and could prove useful to study how human genes function in such tissues *in vivo*. The ability of cell transplantation to create tissues expressing specific genes and gene combinations enables greater insight into the mode by which a protein by itself or in combination cooperates in benign or malignant transformation.

The rationale for the use of bovine cells is mainly due to the low availability of human cells. However, like human cells, bovine cells do not have telomerase activity sufficient for telomere maintenance and therefore undergo telomere shortening, leading to senescence (Thomas et al., 2000). Like human cells, they maintain a stable karyotype under long-term growth in culture. However, they have substantially longer telomeres than human cells (Kozik et al., 1998), enabling greater cell proliferation in the absence of telomerase, both in cell culture and in tissues formed from transplanted cells.

In the first set of experiments that used genetically modified cells, we showed that bovine adrenocortical cells immortalized by the introduction of hTERT (telomerase reverse transcriptase) formed a functional tissue in mice that closely resemble that formed from non-genetically modified cells (Thomas et al., 2000). The tissue formed from the transplanted cells maintained normal growth control. Clearly, enforced telomerase activity in normal adrenocortical cells is not sufficient for transformation and may be a late event in tumor

progression as several reports have documented an increase in telomerase activity in ACCs in comparison to ACAs (Hirano et al., 1998; Mannelli et al., 2000; Else et al., 2008). In subsequent experiments, we showed that bovine adrenocortical cells modified with three genetic changes (hTERT, SV40 large T antigen (SV40 TAg), and oncogenic Ras^{G12V}) were tumorigenic (Thomas et al., 2002) (Fig.1). Mutation at codon 12 in H-Ras impairs its intrinsic GTPase activity and confers insensitivity to cytosolic GTPase-activating proteins, thereby locking the enzyme into an active H-Ras-GTP conformation for signaling through a variety of effector pathways (Shields et al., 2000). Since SV40 TAg binds and inactivates the pRB and p53 tumor suppressor proteins, we concluded that at least the combination of hTERT with mutation in one oncogene and ablation of two tumor suppressor genes is sufficient to fully transform normal adrenocortical cells. Taking advantage of our model of cell transplantation, we were able to study the phenotype of the tissue formed after the transduction of different combinations of our genes. The tissue formed following hTERT and SV40 TAg or SV40 TAg alone is abnormal, yet not tumorigenic. These tissues had a high proliferation rate and a high rate of cell death. Cells expressing oncogenic Ras produced a functional tissue constituted of both clear, lipid-laden cells and eosinophilic lipid-depleted cells with an irregular architecture, cellular pleiomorphism and nuclear atypia. Later on, it has been shown that SV40 TAg and Ras without hTERT were capable of the conversion of both bovine and human adrenocortical cells into malignant cells (Sun et al., 2004).

4. Conclusion

While we believe these experiments are of interest in the context of a multistage model of tumorigenesis, they are also significant in that they provide a proof of principle that the formation of genetically modified tissues by transplantation of adrenocortical cells is feasible. When extended to human carcinoma development, we realized that the SV40 large T antigen expression is obviously not involved in human adrenal tumorigenesis, however this proof of principle let us envision that the experimental model is suitable for checking the transforming potential of genes of interest in tissues and for recapitulating human adrenal tumor initiation and progression. Furthermore, as described above, the model is designed for the evaluation of multiple genetic alterations, individually and in combination thus increasing the ability to mimic the wide spectrum of human ACTs. Studies are underway to assess the genetic alterations observed in human ACTs in our multistage tumorigenesis animal model. Finally, the model should allow further progress in enhancing our understanding into the molecular and cellular mechanisms that underlie the pathogenesis of ACC.

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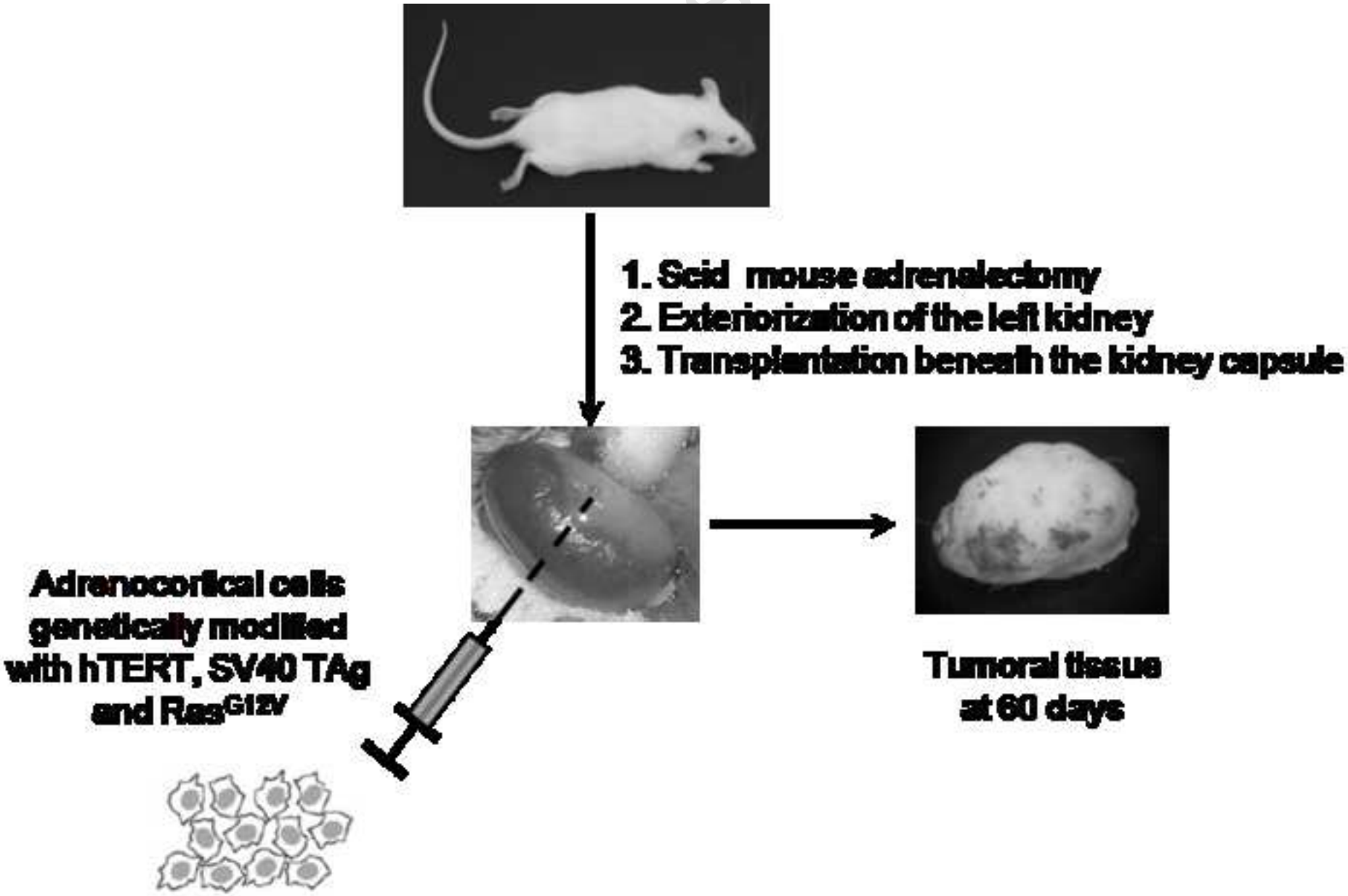
Figure legend

Figure 1: Schematic representation of experimental transplantation of adrenocortical cells. At the time of surgery, the genetically engineered cells are harvested and counted. Each recipient mouse with the scid (severe combined immunodeficiency) mutation is adrenalectomized and the kidney on the left side is exteriorized. Then, $2x10^6$ cells are transplanted beneath the kidney capsule through a transrenal injection with a 50 µl Hamilton syringe fitted with a blunt needle. When the animals are killed, tumoral tissue is readily visible with prominent blood vessels.

Figure


1. Scid mouse adrenalectomy
2. Exteriorization of the left kidney
3. Transplantation beneath the kidney capsule
Adrenocortical cells genetically modified with hTERT, SV40 TAg and Ras^{G12V}
Tumoral tissue at 60 days