In Memoriam: E. Carwile Leroy

ABSTRACT
The search for an animal model of systemic sclerosis (SSc) was tenaciously pursued by E.C. LeRoy. We studied several aspects of the tight skin mouse (Tsk) genetics and pathogenesis under his stimulating influence that contributed to a better understanding of the fibrotic scleroderma-like phenotype of this mouse.

The identification of the fibrillin-1 mutation in the Tsk mouse and the characterization of the cellular and molecular pathways leading to Tsk fibrosis by numerous research groups has opened new avenues in the investigation of human SSc. The enigmatic connections between autoimmunity and ECM homeostasis in fibrotic diseases have received extensive attention in this mouse in which a primary alteration of a connective tissue microfibrilar protein leads to the reproduction of cellular and autoimmune abnormalities strikingly similar to human SSc.

The use of this mouse as a tool to explore anti-fibrotic therapeutic interventions has demonstrated its value in providing useful information on the search for a therapy for this untreatable facet of human disease.

Introduction
Although the search for an animal model of scleroderma (SSc) has been long pursued, the multifaceted pathological picture of the human disease makes the ideal model hard to find (1). SSc pathology includes systemic vascular, immunological and connective tissue abnormalities which are only partially recapitulated by the animal models identified so far. However, the low incidence of the human condition and the elusive nature of the initiating events make particularly attractive the use of animals in order to study the early and sequential pathological events that lead to fibrosis and to test potential pharmacological or molecular interventions. This idea was not dismissed by E.C. LeRoy, who started the search for an immunological model of SSc in the 1980’s in an attempt to understand the links between autoimmune dysfunction as it relates to extracellular matrix (ECM) proteins as a potential link between immunological events and the connective tissue alterations that result in fibrosis. His pioneering work on the response to collagens and other ECM components started a still active line of research that has been renewed in recent years by novel autoantibody specificities such as anti-fibrillin-1 (2, 3).

The connection between the genetic abnormality in the fibrillin-1 gene, which causes the autosomal dominant scleroderma-like disease in the tight skin mouse (Tsk), and the presence of autoantibodies to this protein in the sera from SSc patients remains an enigmatic matter for investigation that also attracted the attention of Dr. LeRoy (4). In the absence of an ideal model for SSc, the component of fibrosis, which is a common response to widely divergent initiating factors, remains the main pathological feature recapitulated by many of the described models. The persistent overexpression of collagens by cultured SSc fibrotic fibroblasts early on described by LeRoy (5) represents the in vitro correlate of the in vivo expansion of a population of dermal fibroblasts with a high content of procollagen mRNA as demonstrated by in situ hybridization studies (ISH) (6). Both approaches have provided a better definition of the SSc-like phenotype in animal models from a cellular perspective. Skin and, in some instances, visceral fibrosis has been described in different murine models in response to chemicals, exogenous growth factors, during graft versus host responses, in several spontaneous or induced mutants (Tsk1 and 2) or in genetically modified mice (Table I) (1, 7). The best studied non-murine model is the UCD 200 chicken, a genetic model that reproduces vascular injury, lymphocytic...
infiltration, and fibrosis (8). However, detailed knowledge of the mouse genome, the wide availability of murine molecular reagents and genetically modified strains represent important advantages that are favoring rapid advances in the knowledge of the murine models of SSc. Although the final effect of increased ECM deposition and fibrosis is induced by widely different cellular and molecular pathways in these models, all converge in an increased population of transcriptionally activated fibroblasts that also characterizes human SSc. Therefore, these models represent useful tools to dissect the possible upstream events as well as to evaluate potential therapeutic tools to ameliorate or reverse fibrosis. In contrast, most of these models only partially reproduce the vascular and immunological alterations observed in human SSc. The vascular hypothesis of SSc that was often explored by Dr. LeRoy under different guises led to his recent formulation of a hypothesis linking infectious agents, vascular lesions and SSc and to one of his latest enthusiastic contributions to this field: the development of a novel animal model of neointimal proliferative lesions, the murine CMV viral infection in immunodeficient mice (9, 10).

Perhaps the best-studied murine model is the Tsk1 mouse, which represents a highly reproducible and predictable model that was caused by spontaneous mutation. Detailed knowledge of the mouse genome has permitted the precise identification of the mutation causing fibrosis in this model, unraveling a novel molecular pathway involved in fibrosis. In addition, the realistic potential of crossing these mice with other mutant or genetically modified strains possessing specific cellular and molecular defects has generated significant advances in the identification of cell types and molecules that potentially participate in fibrosis.

The tight skin mouse genetics

The Tsk1 mouse was first described by Green in 1976 as the result of a spontaneous dominant mutation in the inbred B10.D2 (58N)/Sn strain (11). The phenotype is hallmarked in heterozygous Tsk/+ mice by skin, tendon and cardiac fibrosis and lung emphysema, but lacks other SSc features such as vascular injury or mononuclear cell infiltration (Fig. 1). Homozygous Tsk/Tsk mice die in utero at 7-8 days. The initial studies by Green et al. mapped Tsk 2 CM distal to pallid on chromosome 2.

Fine mapping of the Tsk mutation utilizing classic inter-specific backcross approaches was undertaken by several groups, including our work under the direction of Dr LeRoy, and positioned Tsk between IL1β (interleukin-1β) and β2m (β2-microglobulin) in a region where the Fbn-1 (fibrillin-1) gene had been recently positioned (12, 13). These studies concluded in the identification of partial Fbn-1 gene tandem duplication in Tsk mice by Siracusa et al. in 1996 (14). Fibrillin-1 is the major structural protein of connective tissue microfibrils that are key components of elastic fibers and it is also mutated in another connective tissue disorder, Marfan syndrome. The duplicated fibrillin-1 gene gives rise to an oversized fibrillin-1 protein that results in abnormal microfibrils (15, 16).

Although dissociation between fibrosis and emphysema later suggested that additional factors were involved in the pathogenesis of Tsk emphysema, the direct involvement of abnormal fibrillin-1 in fibrosis has been unequivocally demonstrated by C. Bona and colleagues using a mutated fibrillin-1 transgenic approach (17). The role of the profibrotic growth factors IL-4 and TGFβ, and STAT6 signaling in Tsk fibrosis has also been explored by crossing knockout mice with Tsk and gave support to the hypothesis of an abnormal regulation of fibroblast interactions with the pericellular ECM and local growth factors due to abnormal fibrillin-1 structural and functional (i.e. growth factor binding) properties (18, 19). Fibrillin-1 has domains that are homologous to those of latent TGFβ binding proteins (LTBP) and has been shown to bind these proteins and regulate the activity of TGFβ (20, 21). Consistently, the requirement for other re-

**Table 1. Animal models of SSc.**

<table>
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<tr>
<th>Model Description</th>
<th>Genetic Information</th>
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<tr>
<td>Exposure of mice to vinyl chloride</td>
<td>TGFβ or CTGF subcutaneous injection to mice</td>
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<tr>
<td>Tight skin mouse-1 (Spontaneous mutation in fibrillin-1 gene)</td>
<td>Tight skin mouse-2 (Induced mutation in mouse chromosome 1)</td>
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<tr>
<td>UCD 200 chicken (Spontaneous non-characterized mutation)</td>
<td>Mouse transgenic for mutated collagen β1(1) mutation (collagenase resistant)</td>
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<td>Murine sclerodermatous graft versus host models</td>
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**Fig. 1.** Tight skin mouse phenotype. Externally, the skin fibrosis of the Tsk mouse is evidenced by an indurated and difficult to fold skin similar to human SSc skin. Histologically, a thicker dermis and hypodermis with increased amounts of collagen proteins as stained by eosin (middle panels) or Masson’s trichrome (dark area) (lower panels).
regulatory ECM proteins such as collagen V in the development of Tsk fibrosis has been suggested by Phelps et al., who observed that it could be reversed by genetic complementation with a collagen V-defective mouse (22).

Although the regulatory elements of the procollagen genes involved in transcriptional activation in Tsk mouse have not been completely identified, elegant studies by Denton et al. on the role of a far-upstream enhancer of procollagen I collagen gene using an in vivo transgenic approach revealed that besides TGFβ responsive elements, complex fibroblast-specific and developmentally regulated factors also contribute to the development of fibrosis in this mutant mouse (23).

Therefore, although the molecular mechanisms linking overexpression of procollagen genes and mutated fibrillin-1 have not been fully elucidated, the interpretation of the large body of information generated by this mouse has provided substantial information about the ECM homeostasis and its alterations during fibrosis development.

**Immunological abnormalities in tight skin mouse**

The main difference between the Tsk phenotype and human SSc is the absence of mononuclear cells and overt endothelial lesions in the skin at any phase in the development of skin fibrosis. However, more subtle immunological disturbances paralleling those of human SSc have always been searched for in this mouse. The first abnormality was described by LeRoy in 1983 and consisted in acquired and strong delayed type hypersensitivity to elastase-solubilized lung peptides (2). Although he could not detect antibody responses to these peptides, extensive autoimmunity to a variety of autoantigens, including fibrillin-1, and the presence of fibrillin-1 type II and III in the Tsk mouse repertoire have been recently described, making it feasible that fibrillin-1 peptides were present in the elastin fibers prepared by LeRoy (3).

LeRoy also approached the role of immune cells in Tsk fibrosis through adoptive transfer of Tsk fibrosis using bone marrow or spleen cells transplanted into irradiated syngeneic mice (24). Although he succeeded in transferring fibrosis, the contribution of autoimmunity to the pathogenesis of Tsk fibrosis still remains controversial since the Tsk phenotype fully develops in immunodeficient mice with complete deficiency of T and B cells (SCID mice) (25, 26). A potential explanation that emerges from the current knowledge of mesenchymal stem cells (MSC) is that bone marrow or spleen MSC are very likely transferred together with immunocompetent cells and could eventually home to skin and release abnormal fibrillin-1 in transplanted mice.

These observations have suggested a different interpretation of the significance of autoimmunity in fibrosis as a consequence rather than a cause. In this regard, a striking finding in Tsk mouse was the identification of B cells and autoantibodies against the specific human SSc autoantigens topoisomerase-I and RNA polymerase (27-29). In human SSc, redox damage to these autoantigens has been suggested to underlie changes in their antigenic properties potentially resulting in autoimmunity (30). In the Tsk mouse, although overt vascular lesions leading to ischemia reperfusion damage are not observed, recent data demonstrate a subtle endothelial dysfunction that could contribute to nitric oxide-mediated free radical toxicity (31).

Overall, these observations suggest that either genetic or acquired abnormalities in ECM proteins observed in Tsk or human SSc lead to antigenically modified proteins that elicit autoantibody...
responses. This does not rule out in humans that anti-fibrillin-1 antibodies interact with their target protein and modify their functional properties, leading to an acquired dysfunction mimicking the consequences of the genetically abnormal fibrillin-1 observed in the Tsk mouse and thus contribute to fibrosis.

**Cellular components of tight skin fibrosis**

The only cell types identified in Tsk fibrotic lesions are fibroblasts and mast cells. Cultured Tsk fibroblasts display a phenotype similar to that observed in human SSc cells, with the persistent overexpression of procollagens and ECM proteins (32). Mast cells are present in larger numbers and display increased degranulation, similarly to human SSc (33). This parallelism has allowed for the use of Tsk to sequentially and mechanistically study the behavior of these cells in the pathogenesis of skin fibrosis.

Tsk fibrosis is a highly reproducible process with full penetrance in the heterozygous animal and has a defined temporal pattern of occurrence. The newborn Tsk mouse is indistinguishable from its normal littersmates but at this age, we were able to detect an abnormal regulation of procollagen α1(I) promoter in cultured newborn fibroblasts consistent in an increased basal activity and resistance to further stimulation with TGFβ (Fig. 2) (LeRoy and present authors, unpublished data). The abnormal tensile properties of the intercapular skin that define this phenotype are first detected after the first week of life, progressing thereafter until adulthood. This sequence allowed us to study the series of changes in procollagen gene expression in the different populations of skin fibroblasts during the entire fibrotic process by ISH, with particular focus on the pre-fibrotic phase (34). These studies permitted the identification of a developmentally controlled program of temporospatial regulation of procollagen α1(I), α1(III) and α1(VI) genes expression during post-natal skin development intimately connected to the fibrosis onset.

Tsk is characterized by a failure to downregulate the overexpression of these genes that normally occurs in parallel with the decline in postnatal skin growth after the second week of life (Fig. 3). We suggested that additional changes in the ECM preceding fibrosis should account for the abnormal tensile properties of the skin between the 1st and 2nd post-natal week, and in fact, our further studies detected increased and abnormal fibrillin-1 expression from the 2nd day of life (LeRoy and the present authors, unpublished data). Excessively expressed fibrillin-1 transcripts were composed in similar amounts of the mutated (larger) and normal sized transcripts, suggesting that abnormal heterogeneous microfibrils could be synthesized from birth in Tsk (Fig. 4).

The expression of TGFβ1 mRNA in mouse skin along this time frame revealed a potential role for this factor in postnatal growth but TGFβ1 mRNA was unchanged in Tsk skin, pointing to post-transcriptional mechanisms in the participation of this factor in Tsk fibrosis (34). In this regard, the suggested abnormal fibrillin-1-LTB-TGFβ homeostasis can explain these findings.

In spite of the genetic nature of this program of fibrillar procollagen overexpression in Tsk and the multifactorial influences involved in such a process, partial therapeutic interventions such as anti-IL-4 mAb, synthetic retinoids, or halofuginone have successfully re-verted collagen overexpression and fibrosis in Tsk, opening up some hope for their application to treat human fibrosis (35-37).

The other cell type that attracted Dr LeRoy’s attention in this model was the mast cell (33). His early work on mast cell degranulation in Tsk and its pharmacological modulation by cromoglycate or ketotifen led to the hypothesis of a relevant participation of these cells in Tsk fibrosis (38, 39). Our further studies stimulated by his hypothesis and using Tsk and mast cell deficient mice inter-breeding (double dominant white-spotting mutants W/W”) led to the hypothesis of a secondary role for mast cells in Tsk fibrosis (40). Maximal mast cell infiltration and degranulation was observed in Tsk long after fibrosis onset. However, although mast cells did not initiate fibrosis and were similar in mast cell-deficient Tsk mice until 5-7 months of age, after this point mast cells contributed to a higher degree of fibrosis in mast cell-competent compared to mast cell-deficient Tsk mice. This is in contrast with human SSc, where mast cell degranulation seems to be an early event that precedes skin fibrosis (41). The reasons for mast cell recruitment and degranulation in Tsk and human SSc still remain an enigma. In conclusion, this mutant mouse that attracted Dr LeRoy and his colleagues’ interest has generated a vast array of valuable information and is still open to further research that probably will answer some old and new questions on the pathogenesis of fibrotic diseases.

**References**


