ANTICANCER RESEARCH *25*: 3191-3196 (2005)

Mice Transgenic for *NPM-ALK* Develop Non-Hodgkin Lymphomas

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Abstract. *Background: The t(2;5)(p23;q35) translocation is associated with a high percentage of anaplastic large-cell lymphomas (ALCL) of T- or null-cell phenotype. The translocation produces an 80 kDa hyperphosphorylated chimeric protein (p80) derived from the fusion of the anaplastic lymphoma kinase (ALK) with nucleophosmin (NPM). The NPM-ALK chimeric protein is an activated tyrosine kinase that has been shown to be a potent oncogene and presumably plays a causative role in lymphomagenesis. Materials and Methods: A transgenic mouse line was generated, where the human NPM-ALK cDNA is driven by the lck promoter conferring transgene expression to early T-cells. Results: Mice rapidly developed large cell lymphoblastic lymphomas with a median latency of 8 weeks, primarily involving the thymus, with lymph node as well as histologically evident extranodal organ infiltration by large tumor cells. Conclusion: The transgenic approach described provides direct evidence for the strong transforming potential of NPM-ALK in T-cells and furthermore represents a system for the analysis of the oncogenic events mediated by NPM-ALK in vivo, which might be instrumental in the development of tyrosine kinase inhibitor therapies of potential clinical use.*

Anaplastic large-cell lymphoma (ALCL) is a form of T-cell lymphoma which is defined by CD30 positivity and anaplastic morphology (1). Recently, it has become apparent that there are at least two forms of ALCL based on the presence or absence of a characteristic cytogenetic abnormality, the t(2;5)(p23;q35), which leads to the overexpression of an unusual protein kinase designated *NPM-ALK*. *NPM-ALK* is derived from the fusion of two genes, the gene encoding nucleophosmin *(NPM)* and the *ALK* gene which encodes a tyrosine kinase receptor whose physiological expression is largely limited to neuronal cells (2). *ALK*-positive cases occur more frequently in children and young adults and have a relatively good prognosis. *ALK*-negative ALCL occurs in older individuals and has been associated with a poorer prognosis (3). The N-terminal *NPM* portion of *NPM-ALK* has been shown to mediate dimerization of the molecule, leading to autophosphorylation and activation of the intracellular *ALK* portion containing the tyrosine kinase domain of the receptor. Moreover, since *NPM* participates in nucleocytoplasmic trafficking, the *NPM* portion is responsible for the cytoplasmic and nuclear localization of *NPM-ALK* (4). Among several other fusion partners for *ALK*, *NPM* is the one most often involved in ALCL (5).

We and others have demonstrated that *NPM-ALK* can transform rodent fibroblasts (6, 7) and another study has confirmed that *ALK* protects Ba/F3 cells from interleukin-3 withdrawal (8). Transfer of *NPM-ALK*-transduced bone marrow cells into irradiated host recipient mice has led to the generation of large-cell B-cell lymphomas (9). Subsequently, in recent studies molecular mechanisms of *NPM-ALK*-mediated cellular transformation have been further elucidated. It has been shown that the ALK portion of the fusion protein is absolutely required for transformation (10). Constitutively active ALK fusion proteins can bind to various adaptor proteins and subsequently activate several pathways controlling cell proliferation, transformation and apoptosis. Among those proteins are phospholipase γ (PLC-γ) (10), phosphatidylinositol 3 kinase (PI3K)/Akt (8), Janus kinase 3/signal transducer and activator of

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Key Words: Transgenic mice, NPM-ALK, lymphoma, ALCL.

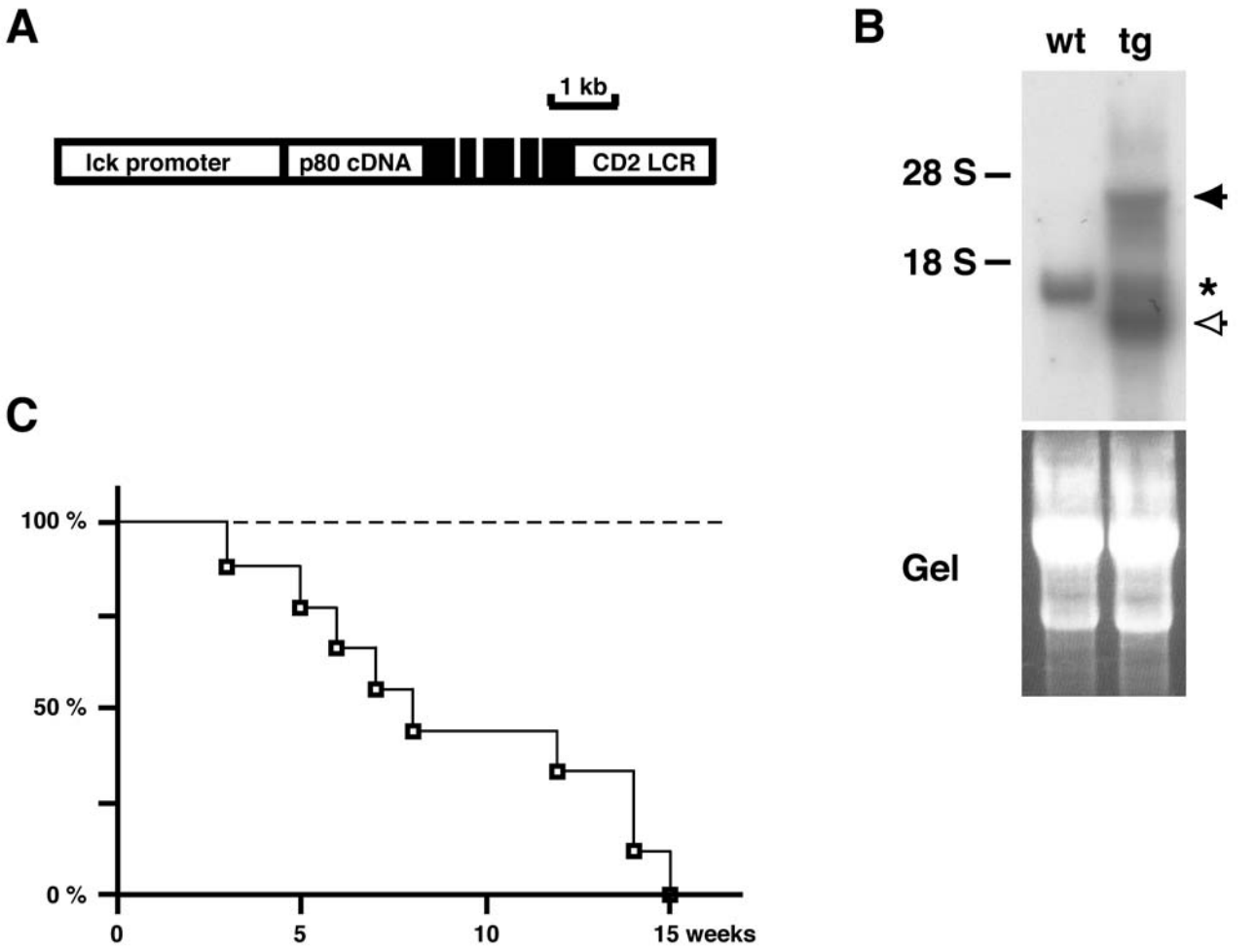

Figure 1. *A. Transgenic construct for the expression of NPM-ALK in T-cells. Open rectangles: lck proximal promoter; p80 cDNA, cDNA encoding the NPM-ALK fusion protein; CD2 LCR, locus control region of the CD2 gene. Filled rectangles: exon sequences from the human growth hormone gene (hGH). The hGH and CD2 LCR sequences are included to confer mRNA stability and integration locus-independent expression, respectively. B. Northern blot analysis of spleen RNA of a wild-type mouse (wt) and a pTLCp80 transgenic mouse (tg). Fifteen μg of total RNA were gelectrophoretically separated and after blotting hybridized to a full-length NPM-ALK cDNA probe. Filled arrow head: NPM-ALK transgene transcript. Asterisk: endogeneous nucleophosmin transcript Open arrow head: truncated transgene transcript. The gel image is shown as a control for RNA quality. C. Kaplan-Meier plot showing the percentage of surviving mice over time. Open squares: pTLCp80 transgenic mice. Dashed line: control mice.*

transcription 3 (Jak3-Stat3) pathways (11). However, all targets and their potential roles have been identified using either non-hematopoietic cells or immortalized B-cells. Therefore, molecular mechanisms of T-cell transformation by ALK chimeras have not been addressed.

With the exception of a recent study by Chiarle *et al.* (12), there has been no direct evidence of the oncogenic potential of ALK chimeras in T-lymphocytes, which represent the most common target of ALK chimeras. To further elucidate the oncogenic potential in an animal model, we generated transgenic mice where *NPM-ALK* is expressed in the T-cell lineage using the lck promoter. Transgenic mice expressing the *NPM-ALK* fusion gene displayed a rapid onset of tumorigenesis. Since tumors displayed characteristics of anaplastic T-cell lymphoma, the transgenic mice generated further proof the oncogenic potential of *NPM-ALK* in T-cells and provide a model to decipher additional oncogenic events involved in disease and potential therapeutic interventions.

Materials and Methods

Generation of transgenic mice. The *Bgl II*-flanked 2.0 kb cDNA encoding the npm-alk fusion was inserted into the singular *BamH I* site of the vector pTLC containing a 3.2 kb lck promoter fragment, the 3' exon-intron structure and poly-adenylation signal of the human growth hormone gene *(2.1 kb)* as well as the locus control

region (2.0 kb) of the human *CD 2* gene (13). Generation of transgenic mice was essentially performed as described (14). Briefly, the construct was digested with *Not1*, and the gel-purified expression cassette was injected into pronuclei of fertilized oocytes from (C57/Bl/6 x DBA) F2 mice and thereafter transferred into the oviducts of pseudopregnant fosters. The transgenic mouse line was then established in the C57/Bl/6 genetic background.

PCR analysis. Genotyping of mice was performed as follows: DNA was extracted from tail biopsies by incubation in 750 µl of tail buffer (50 mM Tris, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% (w/v) SDS, 0.5 mg/ml proteinase K) at 55˚C for 14 hours. After adding 300 µl 5 M NaCl solution, SDS and proteins were precipitated in a microfuge, and DNA was purified from the supernatant by isopropanol precipitation followed by two washes in 500 µl 80% ethanol. Approximately 1 µg of DNA was used for PCR analysis. Primers used to detect the npm-alk allele were p80s: 5'-AGA GGC AAT GAA TTA CGA AGG CAG T-3' and p80as: 5'-AGC AGT AGT TGG GGT TGT AGT CGG T-3'. PCR conditions were: 35 x (45'' 94˚C; 30'' 62˚C; 30'' 72˚C). The PCR product (285bp) was electrophoresed on a 2% agarose gel and photographed using intas (Cologne, Germany) gel documentation equipment.

RNA preparation and analysis. Total RNA was prepared using the guanidinium isothiocyanate-method (15). Fifteen µg of total RNA was loaded onto an 1.2% agarose gel supplemented with 3.3% (w/v) formaldehyde and run in MOPS buffer (20 mM morpholino-propyl-sulphonate, 5 mM sodium acetate, 0.5 mM EDTA, pH 7.0). The RNA was transferred to a nylon membrane (Hybond N^+, Amersham, Braunschweig, Germany) and hybridized to a ^{32}P-labeled full-length NPM-ALK cDNA probe. The filter was washed twice in 2xSSC, 0.1% (w/v) SDS and twice in 1xSSC, 0.1% (w/v) SDS at 65˚C, 10 min each.

Histochemistry and immunohistochemistry. For standard histological analyses, tissue samples were fixed in buffered formalin (10%) for 12 h and subsequently paraffin embedded. Four-µm tissue sections were dewaxed and hematoxylin-eosin-, Giemsa- or PAS-stained, respectively. For immunohistochemistry, sections were microwave-treated for target retrieval (citrate buffer) and incubated with anti-ALK primary antibody (1:700), anti-Ki-67 (Novacastra, Dossenheim, Germany), anti-CD45R0 (1:100) (Novacastra) and anti-CD138 (1:50) (BD Bioscience, Heidelberg, Germany), CD 30 (1:50) (Linaris, Wertheim, Germany). Bound complexes were revealed using the avidin biotin peroxidase complex and a semi-automated immunostainer (DAKO, Hamburg, Germany).

PCR analysis of lymphoma clonality. Genomic DNA was isolated from organs affected with lymphoma by standard procedures. T-cell receptor β and γ (TCRβ and TCRγ, respectively) clonality was assayed by PCR as described by Canela *et al.* (16), using primers specific for the respective vβ, Dβ and Jβ gene segments of the *TCRβ* gene, and primers specific for Vγ4 and Jγ1 of the *TCRγ* gene. PCR products were electrophoresed on a 2 % agarose gel and photographed using intas (Cologne, Germany) gel documentation equipment.

Results

Transgenic mice develop lymphomas. To overexpress the p80/NPM-ALK fusion protein in T-cells, a transgene was constructed containing the npm-alk cDNA under the control of the T-cell-specific lck promoter (17), as depicted in Figure 1A. Micro-injection of the DNA construct into one-cell stage embryos resulted in seven transgenic founder mice. While two of these mice were phenotypically inconspicious and not further studied, five mice showed increased body sizes with body weights between 20% and 100% higher than that of control mice (data not shown). Four of these five founder mice died within 3 months, autopsies revealing thymic, nodal and extranodal lymphoid proliferations. From the remaining transgenic founder mouse, we were able to establish one transgenic mouse line (termed pTLCp80) which could be studied in more detail. Transgene expression was verified by Northern blot using RNA derived from the spleen of a pTLCp80 transgenic mouse and a nontransgenic littermate as a control. As shown in Figure 1B, in addition to the expected 3.5 kb transcript, the transgene gives rise to a truncated transcript, possibly resulting from incorrect splicing or from an internal transcriptional start or stop site. Similar to the individual transgenic founder mice, the transgenic progeny of the pTLCp80 mouse line showed a dual phenotype: an increased body size as compared to nontransgenic littermates (data not shown) and an accelerated mortality due to tumor development. In a cohort of nine transgenic mice all of them died within 15 weeks, whereas control mice remained healthy and alive over the same time period (Figure 1C). Three of the transgenic mice exhibited macroscopically obvious tumors at necropsy.

Characterization of lymphomas. Autopsies of the transgenic mice revealed nodal and extranodal lymphoid proliferations. Histologically, the tumors were mainly classified with thymic and nodal lymphomas, but lymphoid tumors were also detected at extranodal locations, *i.e.* intestine, mammary, lung. The lymphomas were primarily composed of medium-sized lymphoblasts (Figure 2A, B) with a very high proliferation index (about 40-50%) as revealed by Ki-67 immunohistochemistry (Figure 2C). Immunohistochemically the lymphomas were of T-cell phenotype positive for CD 45 R0 and negative for CD 138 (Figure 2H, F). A large fraction was also positive for CD 30 (Figure 2E). Tumor cells were not only positive for *NPM-ALK* on a transcriptional level, as demonstrated by RT-PCR (data not shown), but also on the translational level, as revealed by immunohistochemistry (Figure 2D). The latter underscored an ALCL-typical sinusoidal growth pattern within lymph nodes in some cases (Figure 2D). T-cell clonality analysis using PCR revealed a clonal situation (Figure 2G).

Discussion

We have produced and characterized a mouse model of *NPM-ALK*-induced lymphomagenesis with a vector

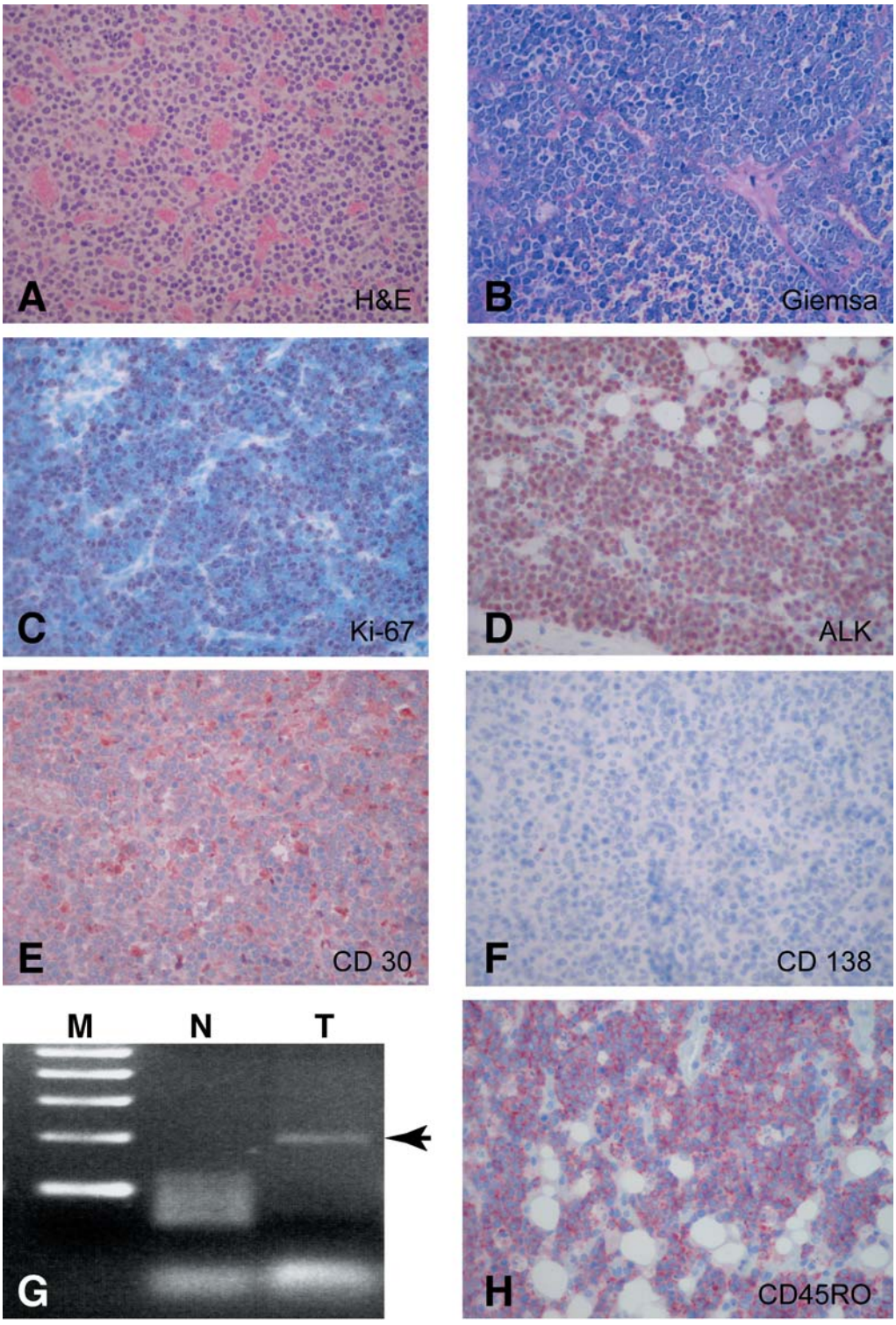

Figure 2. *Characterization of tumors. Shown are paraffin sections of tumor material stained with hematoxylin-eosin (A) and Giemsa (B). C-F, H. Immunohistochemical stainings (red color) of depicted antigens. C. Immunohistochemical staining of Ki-67 antigen revealing proliferative cells. D-F. Immunohistochemical staining of ALK (D), CD 30 (E) and CD 138 (F). H. tumor section stained for CD45RO. G. Example of TCR clonality analysis: PCR was performed using primers specific for $V\beta_6$ and $J\beta_1$ of the TCRβ gene. Lane M: DNA standard (100 bp ladder). In contrast to lane N where amplification of lymphocyte DNA from a normal mouse resulted in multiple bands, in DNA of a tumor from a transgenic mouse (lane T) one specific fragment is amplified, indicative of a clonal origin of the tumor.*

containing the lck promoter which had been previously shown to be active in T-cells (13). This novel approach clearly demonstrated that human *NPM-ALK* invariably leads to the generation of T-cell lymphomas, a matter of debate even after several *in vitro* studies had demonstrated the transforming capacity of the *NPM-ALK* chimeric gene in a variety of cell types, *i.e.* rat1 fibroblasts (6). As an *in vivo* approach, Kuefer *et al.* (9) have developed a retro-viral system to demonstrate the ability of *NPM-ALK* to transform hematopoietic cells to large B-cell lymphomas. Recently, a transgenic model has been described where overexpression of *NPM-ALK* in hematopoietic cells caused B-cell lymphoma (18). However, since most *NPM-ALK*-positive tumors are ALCL with a T-cell phenotype (1), only an *in vivo* T-cell model is suitable for studying the role of *NPM-ALK* and potential treatment options. Therefore, transgenic mice, where the fusion of *NPM/ALK* is expressed in the T-cell lineage, were generated using the lck promoter.

Our experiments revealed that constitutive expression of *NPM-ALK* leads, with a very short latency, to spontaneous lymphomagenesis in mice. Together with recent results from Chiarle *et al.* (12), this finding underscores the particularly potent transforming capacity of *NPM-ALK* in comparison to other oncogenes that have been studied in similar experiments so far. The even more rapid lymphomagenesis in our model compared to that of Chiarle *et al.* is possibly due to the lck promoter which confers transgene expression at earlier stages of T-cell development than the CD4 promoter used by Chiarle *et al.* (19, 20).

The tumors observed were of immature lymphoblastic type. This may reflect that in both studies the transgene was turned on very early in individual development, which does not reflect the human situation of classic *NPM/ALK*-related ALCL development, where the majority of patients is of older age (21). The physiologically high turn-over rate of the cell compartments addressed by the transgene promoters may, in addition, help accumulate additional genetic alterations required to support ALK transformation.

This rapid onset of lymphoma development reflects the very redundant and complex pleiotropic mechanism of *NPM-ALK*-induced cell transformation. Studies dealing with *NPM-ALK*-related signaling pathways have shown, that ALK can simultaneously activate PI3 kinase- and PLC γ-related pathways and trigger either inhibition of apoptosis or activate cell proliferation (8, 10, 11). We also have shown previously that ALK can activate cyclin D and c-myc (6) by the effector STAT 3 (11).

Very surprisingly, all transgenic mice were, from early on, considerably larger than their littermates. This phenomenon, which had not been observed in the study by Chiarle *et al.* (12), may be due to a very high turn-over rate of T-cells, which probably cause a deregulated non-physiological expression of various cytokines or growth-factors. Alternatively, an unexpected splicing event may have resulted in the expression of a functional hGH portion from the hGH sequences included in the transgene construct. The truncated transcript observed in our Northern analysis may argue in favor of the latter hypothesis. However, other transgenic mice generated with a similar vector have not shown this effect (13, 22).

In conclusion, our findings confirmed the tumorigenic activity of human *NPM-ALK in vivo*. Our potent model will provide a valuable tool for further elucidation of *NPM-ALK* signalling and for the identification of new putative recurrent aberrations cooperating with ALK in promoting T-cell transformation *in vivo*. This knowledge may result in treatment options that can then be tested using the transgenic mice described.

Acknowledgements

We thank Wiebke Jeske and Mathilde Hau-Liersch for their technical assistance and Falk Weih for providing the pTLC vector. A special thanks to Gerrit Klemm and his team for processing and printing. This work was supported by grants from the Mildred Scheel Stiftung and Herbert Reeck Stiftung to H.S. and A.W. (Grant # 10-2002-We 2).

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Received February 16, 2005
Accepted May 4, 2005