Human adenoviruses (HAdV) encode several proteins with multifunctional character, which are supportive for and/or capable of transforming primary rodent cells (Branton et al., 1985; Graham, 1984; Nevins & Vogt, 1996; Ricciardi, 1995). The oncogenic determinants of the model system HAdV type 5 are located within the early region 1 (E1) and early region 4 (E4) of the viral genome. Whereas the adenovirus type 5 (Ad5) E1A and E1B proteins are definitely required for oncogenic transformation (Branton et al., 1985; Graham, 1984; Nevins & Vogt, 1996; Ricciardi, 1995) mediating cell cycle progression (reviewed by Gallimore & Turnell, 2001) and concurrent inhibition of tumour suppressor proteins (Yew et al., 1994), additionally expressed proteins of the E4 region substantially increase the transforming potential by operating through a complex network of cellular regulatory components (reviewed by Täuber & Dobner, 2001a).

Since the initial description of adenovirus type 12 (Ad12) inducing tumorigenicity in newborn rodents (Trentin et al., 1962) most of the viral genes involved in adenovirus transformation are now known, yet it still remains unclear as to why human adenoviral gene products are highly oncogenic in rodent, but not in human primary cells. With few exceptions (Byrd et al., 1982; Fallaux et al., 1996, 1998; Gallimore et al., 1986; Graham et al., 1977; Schiedner et al., 2000; van den Heuvel et al., 1992; Whittaker et al., 1984), attempts to transform primary human cells in culture by using different types of adenovirus/subgenomic DNA fragments have turned out to be unsuccessful or extremely inefficient, whereas rodent cells can be efficiently transformed (reviewed by Branton et al., 1985; Endter & Dobner, 2004; Graham, 1984; Graham et al., 1984). As it has been suggested that lagomorphs and rodents form a monophyletic group (glires) and are therefore closely related (Fig. 1a), we analysed the transformation capabilities of the Ad5 oncogenic determinants in cells of an additional order (Lagomorpha) of the higher mammals (Fig. 1a). Therefore, we used primary rabbit lens epithelial (pRLE) cells as they represent an easily accessible, reliable and reproducible source of primary rabbit cell material. The cells were transfected by calcium phosphate (Graham & van der Eb, 1973) with pXC15 plasmid (Logan et al., 1984) encoding the Ad5 E1A/E1B proteins, alternatively including pcDNA3(neo)-E4orf5 (Rubenwolf et al., 1997), pcDNA3(neo)-E4orf5 (Nevels et al., 1999b) or empty vector pcDNA3(neo). As previously published for primary rat cells (Nevels et al., 1997), ongoing cultivation of transfected cells for ~3 weeks leads to the elimination of untransformed cells as a consequence of senescence and cell death, whereas transformed cells form multi-layered cell condensations (foci). Fig. 1(b) shows representative results of focus forming assays of pRLE cells in comparison...
permanent cell lines by G418 selection. With crystal violet. Alternatively, stably transformed polyclonal and tively empty vector pcDNA3(neo)

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Furthermore, it appears that adenoviral induced focus growing much slower and to lower cell densities.

E1A/E1B-derived RLE foci (AB RLEC) exhibit a morpho-

tual repression (Yew et al., 1994) and nucleo-cytoplasmic

E1A/E1B/E4orf6 (ABS cells) and Ad5 E1A/E1B/E4orf3 (ABT cells) were established by G418 selection. As expected, analysis of representative cell lines by phase microscopy highlight significant morphological alterations compared with primary and/or spontaneously immortalized RLE cells (Fig. 2a). Consistently with already published data for transformed BRK cells (Nevels et al., 1999a), the RLE cell lines (AB1-2, ABS1-6 and ABT2-4) appear considerably smaller and grow in densely packed accumulations. As continuous adenoviral gene expression is considered to be a pre-requisite for maintenance of a transformed phenotype, we screened for the transfected Ad5 proteins by Western blot analysis (Fig. 2b). Therefore, the expressed proteins were visualized by subsequent steps of total cell extract preparation, SDS-PAGE, immunoblotting and sequential antibody incubation, as described previously (Kindsmüller et al., 2009). As expected, primary as well as spontaneously immortalized RLE cells did not show any detectable amounts of viral proteins (Fig. 2b; lanes 3 and 4), whereas the rat cell line ABS1 (Nevels et al., 1997, 1999a) and human cell line 911 (Fallaux et al., 1996) expressed the adenoviral E1A, E1B and E4orf6/E1A and E1B proteins (Fig. 2b; lanes 1 and 2), respectively. It is well known that transcriptional repression of the tumour suppressor protein p53 is an integral part in facilitating complete cell transformation by adenoviral oncogenes (Yew & Berk, 1992; Yew et al., 1994). Although, E1A induces stabilization of p53 during metabolic cell stimu-

lation (Gallimore & Turnell, 2001; Lowe & Ruley, 1993), E1B-55K leads to complete repression of p53-mediated transcripational activation via subsequent steps of inter-

action (Kao et al., 1990; Sarnow et al., 1982), transcriptional repression (Yew et al., 1994) and nucleo-cytoplasmic relocalization (Endter et al., 2001, 2005). Consistently, AB1-2 RLE (Fig. 2b; lane 5) and ABT2-4 RLE (Fig. 2b; lane 7) cells showed enhanced p53 levels compared with the negative controls (Fig. 2b; lanes 3 and 4), whereas the

to primary baby rat kidney (pBRK) cells. Although no foci could be observed in the corresponding negative controls, the Ad5 E1A/E1B proteins induce substantial focus formation in pBRK cells synergistically enhanced by E4orf6, leading to multi-layered cell aggregates within 21 days (Fig. 1b; upper panel). Therefore, we could show for the first time that adenoviral oncogenes induce substantial alterations in morphology and growth be-

haviour in pRLE cells (Fig. 1b; lower panel) exhibiting a comparable but distinct phenotype to fully transformed BRK cells (Fig. 1b; upper panel). In direct comparison, E1A/E1B-derived RLE foci (AB RLEC) exhibit a morphology similar to E1A partially immortalized BRK foci growing much slower and to lower cell densities. Furthermore, it appears that adenoviral induced focus formation occurs at least two to three times less frequently in pRLE than in pBRK cells, indicating a reduced transformation efficiency of the E1 and E4 proteins within the rabbit cells. Consistently, the assumption that stably transfected RLE cells exhibit a delay in cell growth and/or macroscopic cellular transformation appears adequate as foci development took at least 10 weeks to reach the size shown (Fig. 1b; lower panel), whereas foci derived from pBRK cells were evident after 3 weeks. In contrast to previously published results for rat cells (Nevels et al., 1997, 1999a, b; Täuber & Dobner, 2001a, b), concurrent expression of the adenoviral E4orf6 protein in pRLE cells does not significantly increase the transformation capabilities of the E1 proteins per se. Currently, the molecular reason for this observation remains elusive; however, it is tempting to speculate that rabbit cells might exhibit a comparable phenotype previously described for human cells, highlighting the anti-proliferative functions of con-

tinuous E4orf6 expression (Catalucci et al., 2005; Hart et al., 2006). Alternatively, several polyclonal cell lines from cultures transfected with Ad5 E1A/E1B (AB cells), Ad5 E1A/E1B/E4orf6 (ABS cells) and Ad5 E1A/E1B/E4orf3 (ABT cells) were established by G418 selection. As expected, analysis of representative cell lines by phase microscopy highlight significant morphological alterations compared with primary and/or spontaneously immortalized RLE cells (Fig. 2a). Consistently with already published data for transformed BRK cells (Nevels et al., 1999a), the RLE cell lines (AB1-2, ABS1-6 and ABT2-4) appear considerably smaller and grow in densely packed accumulations. As continuous adenoviral gene expression is considered to be a pre-requisite for maintenance of a transformed phenotype, we screened for the transfected Ad5 proteins by Western blot analysis (Fig. 2b). Therefore, the expressed proteins were visualized by subsequent steps of total cell extract preparation, SDS-PAGE, immunoblotting and sequential antibody incubation, as described previously (Kindsmüller et al., 2009). As expected, primary as well as spontaneously immortalized RLE cells did not show any detectable amounts of viral proteins (Fig. 2b; lanes 3 and 4), whereas the rat cell line ABS1 (Nevels et al., 1997, 1999a) and human cell line 911 (Fallaux et al., 1996) expressed the adenoviral E1A, E1B and E4orf6/E1A and E1B proteins (Fig. 2b; lanes 1 and 2), respectively. It is well known that transcriptional repression of the tumour suppressor protein p53 is an integral part in facilitating complete cell transformation by adenoviral oncogenes (Yew & Berk, 1992; Yew et al., 1994). Although, E1A induces stabilization of p53 during metabolic cell stimu-

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lower p53 levels in ABT2-4 RLE correlated with the lower levels of E1A expression (Fig. 2b; lane 7) (Gallimore & Turnell, 2001; Lowe & Ruley, 1993). During viral infection, the E1B-55K and E4orf6 proteins form an SCF-like E3-ubiquitin ligase complex (Blanchette et al., 2004) inducing the ubiquitin-dependent proteasomal degradation of cellular target proteins such as p53 (Querido et al., 1997, 2001a, b), mre11 (Stracker et al., 2002), DNA ligase IV (Baker et al., 2007) and integrin 3α (Dallaire et al., 2009). It has been suggested that E1B-55K/E4orf6-dependent degradation of the tumour suppressor p53 might also be important for cellular transformation explaining the remarkably enhanced focus forming activity of E1A/E1B/E4orf6 in primary BRK cells compared with E1A/E1B-55K alone (Fig. 1b; upper panel) (Nevels et al., 1997). Interestingly, this previously described phenomenon of hyper-transformation by E4orf6 could not be observed in RLE cells (Fig. 1b; lower panel) correlating with only moderately reduced p53 levels in ABS1-6 (Fig. 2b; lane 6) compared with AB1-2 (Fig. 2b; lane 5). Although the results shown are representative for several isolated clones (data not shown) and are consistent with previously made observations of established adenoviral transformed BRK cell lines (Nevels et al., 1999a, b), it is likely that the molecular mechanism and efficiency of E1B-55K/E4orf6-mediated p53 degradation in rabbit cells depends on various factors such as cell type origin, species origin and adenoviral protein levels. In contrast to immunofluorescence analysis (data not shown), it was not possible to detect any amounts of E4orf3 within the ABT2-4 RLE cell line by Western blot analysis implying low expression of the adenoviral protein. To further substantiate the assumption that Ad5 E1A, E1B and E4orf6/E4orf3 are capable of full oncogenic transformation of primary rabbit cells in vitro, we performed detailed analysis of several criteria such as growth behaviour, tumour induction in vivo and histology of tumour samples (Fig. 3). For growth studies, 3 × 10⁴ cells were initially plated onto six-well dishes in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum (FCS). The medium was replaced every 48 h and viable cells from duplicated dishes were counted by using an improved Neubauer counting chamber. Promoted by the expression of the adenoviral proteins, all established RLE cell lines showed significantly higher growth rates than pRLE cells ending up in a cell number at least 15–20 times higher (Fig. 3a). Finally, 1 × 10⁶ cells of each cell line were resuspended in serum-free DMEM and subcutaneously...
injected into NMRI(nu/nu) mice (six animals per group) with a mean body weight of 20 g. Tumour growth kinetics were recorded by weekly measurement of tumour diameters at the inoculation site (thoracic mammary fat pad), whereas tumour areas were calculated as the product of two perpendicular diameters. For ethical reasons the mice were killed after the tumours reached a size of approximately 300 mm$^2$ or 6 weeks after injection. During an observation period of 22 days, all animals who received the established RLE cell lines developed visually apparent, rapidly growing tumours, while simultaneously none of the animals given pRLE cells developed any kind of detectable tumour (Fig. 3b). Consistent with previous published results for BRK cells (Nevels et al., 1997), tumours induced by ABS1-6 RLE cells containing Ad5 E4orf6 showed a rapid, hyper-transformed phenotype inducing large tumours with sizes of about 49.5–93.5 mm$^2$ covering an area at least two to three times higher than AB1-2 or ABT2-4 (Fig. 3b). Supporting our results, additionally performed histopathological analysis revealed more characteristics of uncontrolled cellular growth induced by the adenoviral transformed RLE cells (Fig. 3c). ABS tumour samples resembled the aspect of highly malignant adenocarcinoma exhibiting a well vasculated tissue consisting of undifferentiated cells densely packed within the tumour [Fig. 3c; image (iii)]. Whereas ABT2-4 induced tumours also showed some of these characteristics too [Fig. 3c; image (iii)], AB1-2 induced tumours [Fig. 3c; image (i)] are characterized by a much higher content of stroma, necrotic areas as well as polyploid cells. Depending on the analysed histological sections, several specific characteristics of malignant cell growth such as polyploid cells, tripolar cell-division spindles and blood vessels were evident [Fig. 3c; images (i)–(iii); indicated by arrows]. Taken together, these in vitro and in vivo studies clearly demonstrate that the established RLE cell lines exhibit a fully transformed phenotype mediating extensive cell growth and tumourigenicity in nude mice.

Despite extensive studies of the adenoviral oncogenes encoded by E1 and E4, it has remained elusive as to how these adenoviral proteins facilitate full transformation of primary rodent cells, while concurrently failing to do so in human cells in vitro. Several models have been discussed involving deregulation of cellular proliferation (Gallimore & Turnell, 2001), repression of tumour suppressor proteins (Yew et al., 1994) and induction of genomic instability by the modulation of the DNA damage response (Härtl et al., 2001; Stracker et al., 2002) or by adenoviral proteins per se (Nevels et al., 2001). However, by using pRLE cells we were able to demonstrate that the transforming potential of adenoviral proteins is not exclusively restricted to the mammalian order Rodentia, but also seems to include cells of the closely related order Lagomorpha of the higher mammals. Furthermore, the established RLE cell lines may represent a suitable tool after further development to generate E4-mutated adenoviruses, which has so far been difficult as mutations within the E4 region often prove to be lethal without an adequate helper-cell system providing
these functional factors in trans (Catalucci et al., 2005; Hart et al., 2006).

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