



Cadherin-6 Mediates Axon-Target Matching in a Non-Image-Forming Visual Circuit

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SUMMARY

Neural circuits consist of highly precise connections among specific types of neurons that serve a common functional goal. How neurons distinguish among different synaptic targets to form functionally precise circuits remains largely unknown. Here, we show that during development, the adhesion molecule cadherin-6 (Cdh6) is expressed by a subset of retinal ganglion cells (RGCs) and also by their targets in the brain. All of the Cdh6-expressing retinorecipient nuclei mediate non-image-forming visual functions. A screen of mice expressing GFP in specific subsets of RGCs revealed that Cdh3-RGCs which also express Cdh6 selectively innervate Cdh6-expressing retinorecipient targets. Moreover, in Cdh6-deficient mice, the axons of Cdh3-RGCs fail to properly innervate their targets and instead project to other visual nuclei. These findings provide functional evidence that classical cadherins promote mammalian CNS circuit development by ensuring that axons of specific cell types connect to their appropriate synaptic targets.

INTRODUCTION

Functional neural circuits consist of precise connectivity between specific sets of neurons. The assembly of such circuitry often requires that axons bypass numerous targets before selectively terminating in just one or a few specific targets. Over the last century, much progress has been made in understanding how axons undergo directed growth and pathfinding and how they form topographic maps (Sperry, 1963; Dickson, 2002; Feldheim and O'Leary, 2010). How mammalian axons identify which targets to innervate, however, remains poorly understood.

The axonal connections formed by the eyes with the brain are an attractive model for exploring mechanisms of axon-target

recognition in the mammalian CNS. Retinal ganglion cells (RGCs) are the output neurons of the eye and they are divided into ~20 different types. Each RGC type encodes a different quality of the visual scene, such as brightness, direction of motion or edges (Masland, 2001; Berson, 2008), and sends that information to a limited number of retinorecipient targets that in turn regulate specific aspects of perception and behavior. For example, type 1 melanopsin RGCs encode the level of environmental illumination and project to the hypothalamic suprachiasmatic nucleus and midbrain olivary pretectal nuclei, which are involved, respectively, in entrainment of circadian rhythms and pupillary reflexes (Ecker et al., 2010; Güler et al., 2008). In contrast, the axons of alpha and On-Off direction selective RGCs innervate the dorsal lateral geniculate nucleus (dLGN) and the superior colliculus (SC) (e.g., Bowling and Michael, 1980; Tamamaki et al., 1995; Huberman et al., 2008, 2009; Rivlin-Etzion et al., 2011), targets involved in pattern vision and visually guided gaze shifts.

What mechanisms enable CNS axons to connect to specific targets and to avoid others? In the developing *Drosophila* visual system, adhesion plays a critical role in axon-target matching (Clandinin and Feldheim 2009). The cadherins are a family of molecules hypothesized to establish precise CNS connectivity by promoting selective adhesion among neurons expressing the same cadherin or combination of cadherins (Takeichi, 2007). Previous work showed that N-Cadherin is important for targeting specificity of *Drosophila* photoreceptors: loss of function mutations and experiments with genetically mosaic animals demonstrated that N-cadherin is required both in photoreceptors R1-R6 and in their target lamina neurons (Lee et al., 2001; Prakash et al., 2005). In chick, antibodies against N-cadherin disrupt laminar specific RGC axon targeting in vitro (Inoue and Sanes, 1997). Whether cadherins regulate axon-target matching in the mammalian CNS, however, remains unknown.

Here, we show that Cadherin-6 (Cdh6) is expressed by a subset of RGCs and by their retinorecipient targets in the brain, all of which mediate non-image-forming visual functions. We also show that Cdh3-GFP and Cdh6-GFP transgenic mice label the RGCs that innervate Cdh6 expressing targets. We then provide

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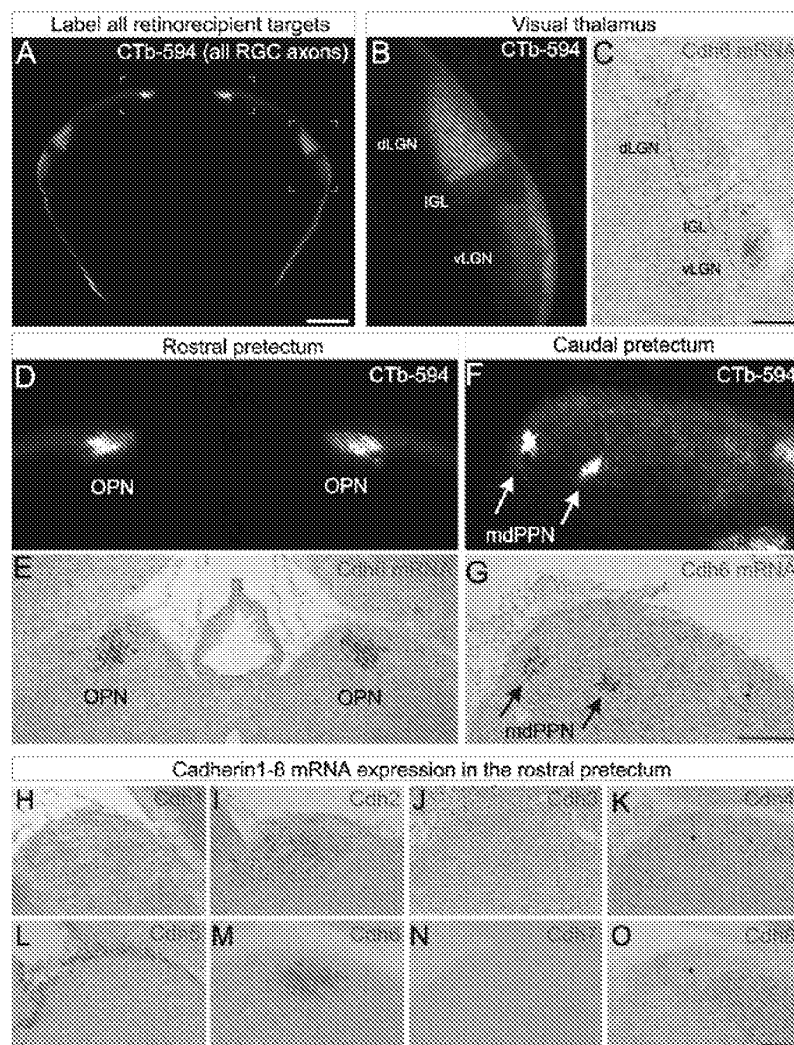


Figure 1. Cadherin-6 Is Expressed in Specific Subcortical Visual Nuclei

(A) CTb-594 labeled RGC axons at the forebrain-midbrain border of a postnatal day 2 (P2) mouse. Image is in coronal plane. Bracketed regions correspond to panels (B and D). Scale = 500 μ m. (B) Pan-RGC axon labeling in the visual thalamus. The ventral lateral geniculate nucleus (vLGN), intergeniculate leaflet (IGL) and dorsal lateral geniculate nucleus (dLGN) contain RGC axons. (C) Cdh6 mRNA expressing cells in the IGL and vLGN. Scale = 100 μ m. (D) CTb-594 labeled RGC axons in the rostral pretectum. The left and right olivary pretectal nuclei (OPN) are densely innervated. (E) Cdh6 mRNA expression in the OPN of a P1 mouse. (F) RGC axons in the caudal pretectum. The medial division of the posterior pretectal nucleus (mdPPN) of Scalia (1972) appear as two foci (arrows). (G) Cdh6 mRNA in the mdPPN (arrows) of a P1 mouse. Asterisk: a few Cdh6 expressing cell; these may correspond to the caudal-most OPN. Scale = (D)–(G), 250 μ m. (H–O) Cdh1–8 antisense mRNA labeling in the rostral pretectum of the early postnatal mouse. Cdh2 (I) and Cdh6 (M) are expressed by the OPN, whereas Cdh4 (J) and Cdh8 (O) are expressed by cells nearby the OPN but not in the OPN itself (asterisks). Cdh1 (H), 3 (J), 5 (L), and 7 (N) are not expressed by the OPN or nearby nuclei but some of these (e.g., Cdh7) are expressed by other retinorecipient targets (not shown). (H–O) Scale = 150 μ m. See also Figure S1.

targets that mediate non-image-forming visual functions: the thalamic ventral lateral geniculate nucleus (vLGN), the intergeniculate leaflet (IGL) (Figures 1B and 1C), the olivary pretectal nuclei (OPN) (Figures 1D, 1E, and 1M) and the medial division of the posterior pretectal nucleus (mdPPN) (Figures 1F and 1G). The Cdh6-expressing targets relate to circadian rhythm entrainment (vLGN and IGL) (Harrington, 1997), pupil constriction

(OPN) (Güler et al., 2008) and oculomotor functions (mdPPN) (Gioli et al., 2006). Cdh6 expression was specific to these targets during late embryonic and early postnatal development (~E18–P4), the stage when RGC axons innervate their targets (Gode-ment et al., 1984) with Cdh6 expression persisting into the first postnatal week (Figure 1). The other cadherins we assayed showed patterns of expression that were notably different from Cdh6. Cdh1, 3, 4, 5, 7, and 8 were not expressed by the OPN or mdPPN although Cdh4, 7, and 8 were expressed by other retinorecipient nuclei (Figures 1H, 1J, 1K, 1L, 1N, and 1O and unpublished observations). Indeed, Cdh4 and Cdh8 were expressed by regions adjacent to and surrounding the OPN, but were absent from the OPN itself (Figures 1K and 1O). Of the cadherins we assayed, only one of them, Cdh2, was expressed by the OPN during early postnatal development, but Cdh2 was expressed by all other retinorecipient areas too (Figure 1I; data not shown). Thus, during the developmental stage when RGC axons

RESULTS

Cadherin-6 Is Expressed by a Subset of RGCs and Non-Image-Forming Visual Targets

As a first step toward assessing the role of cadherins in mammalian visual circuit assembly, we analyzed the expression patterns of several classical cadherins in the mouse brain. We visualized retinorecipient targets by making bilateral intravitreal injections of cholera toxin beta conjugated to Alexa 594 (CTb-594) which labels all RGC axons, and then compared each CTb-594 labeled target in the brain with the mRNA expression patterns of cadherin 1 (Cdh1), Cdh2, Cdh3, Cdh4, Cdh5, Cdh6, Cdh7, and Cdh8. We took particular notice of Cdh6 because it was highly and selectively expressed by a subset of the retinorecipient

(OPN) (Güler et al., 2008) and oculomotor functions (mdPPN) (Gioli et al., 2006). Cdh6 expression was specific to these targets during late embryonic and early postnatal development (~E18–P4), the stage when RGC axons innervate their targets (Gode-ment et al., 1984) with Cdh6 expression persisting into the first postnatal week (Figure 1). The other cadherins we assayed showed patterns of expression that were notably different from Cdh6. Cdh1, 3, 4, 5, 7, and 8 were not expressed by the OPN or mdPPN although Cdh4, 7, and 8 were expressed by other retinorecipient nuclei (Figures 1H, 1J, 1K, 1L, 1N, and 1O and unpublished observations). Indeed, Cdh4 and Cdh8 were expressed by regions adjacent to and surrounding the OPN, but were absent from the OPN itself (Figures 1K and 1O). Of the cadherins we assayed, only one of them, Cdh2, was expressed by the OPN during early postnatal development, but Cdh2 was expressed by all other retinorecipient areas too (Figure 1I; data not shown). Thus, during the developmental stage when RGC axons

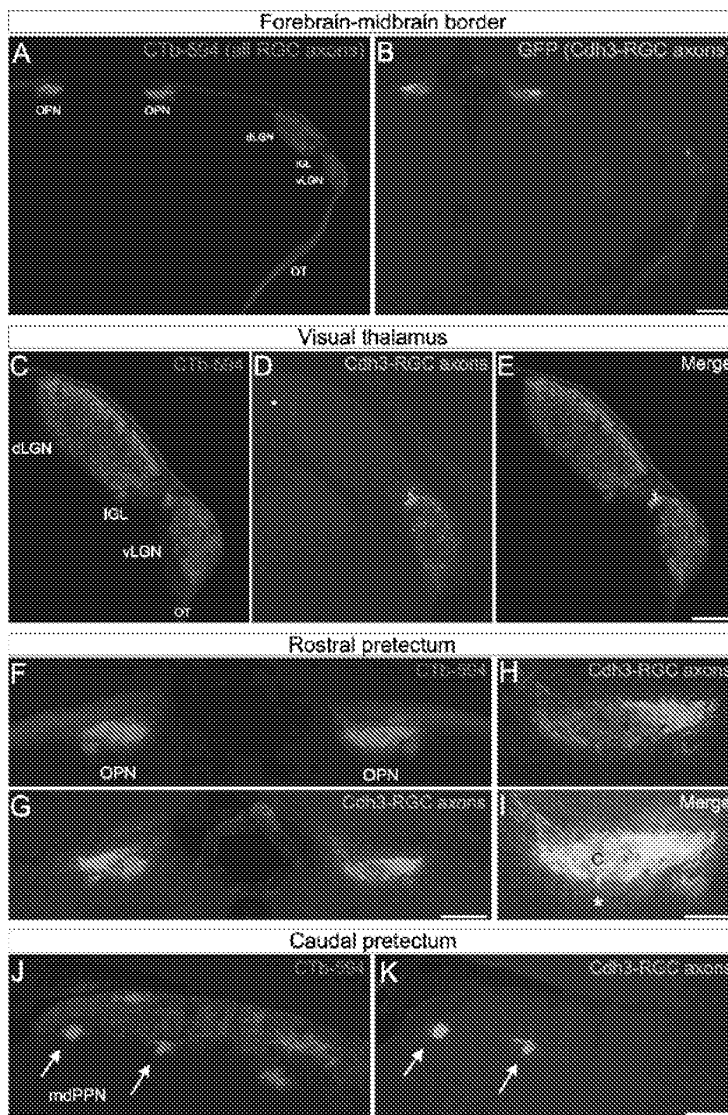


Figure 2. Cdh3-RGCs Project to Cdh6-Expressing Visual Targets

(A) CTb-594 labeled RGC axons and (B) Cdh3-GFP⁺ RGC axons ("Cdh3-RGCs") at the level of the forebrain-midbrain border (coronal plane). The optic tract (OT), vLGN, IGL, dLGN, and OPN are apparent from CTb-594 label. (B) Cdh3-RGC axons in the OT, IGL, vLGN and OPN. Scale = 500 μ m. (C-E) CTb labeled RGC axons (C), and Cdh3-RGC axons (D), and their merge (E) in the visual thalamus. Except for sparse terminals in the dorsal cap of the dLGN (asterisk), Cdh3-RGC axons selectively terminate in the vLGN and IGL. Scale = 300 μ m. (F-I) Rostral pretectum OPN with (F) CTb-labeled RGC axons and (G and H) Cdh3-RGC axons and (I) their merge. (I) The OPN core ("c") is occupied by Cdh3-RGC axons, whereas the OPN "shell" (asterisk) is not and contains only CTb-594 label. Scale in (F) and (G) = 250 μ m; scale in (H and I) = 100 μ m. (J and K) The caudal pretectum with (J) CTb-labeled RGC axons and (I) Cdh3-RGCs axons. (J) The caudal pretectum includes many retinorecipient areas, including the mdPPN (arrows). (K) Cdh3-RGC axons selectively terminate in the mdPPN (arrows). (J and K) Scale = 175 μ m. See also Figures S1-S3.

those targets for the axons of Cdh3-GFP RGCs (hereafter referred to as Cdh3-RGCs). Cdh3-RGC axons terminated in the vLGN and IGL, whereas the adjacent dLGN, the target that relays visual information to the cortex for image perception, was virtually devoid of Cdh3-RGC axons (Figures 2A-2E and S1). Cdh3-RGC axons also densely innervated the OPN (Figures 2A, 2B, 2F-2I, and S1) specifically in the OPN "core," whereas the OPN "shell" was devoid of Cdh3-RGC axons (Figures 2H and 2I). A limited number of Cdh3-RGC axons remained in the optic tract until they arrived to the caudal pretectum, wherein they terminated in two dense foci corresponding to the mdPPN (Figures 2J and 2K; Scalia, 1972).

We are confident the GFP axons observed in the vLGN, IGL, OPN, and mdPPN originated from RGCs because they disappeared from those targets following eye removal (not shown). Indeed, with the exception of olfactory glia, a

subset of brainstem nuclei and a small population of cells near the fourth ventricle, the brains of Cdh3-GFP mice were remarkably devoid of GFP-expressing cells (Figures 2A, 2B, S1, and S2). We found no evidence of Cdh3 mRNA expression in any retinorecipient targets (Figures 1J and S2). Together, our data indicate that Cdh3-GFP mice selectively label the RGCs that project to the vLGN, IGL, OPN, and mdPPN, the very same non-image-forming retinorecipient targets that express Cdh6.

Cdh3-RGCs Selectively Innervate Cdh6-Expressing Visual Targets in the Brain

To examine whether Cdh6 plays a functional role in retinofugal targeting, we needed a way to visualize the axons of the particular RGCs that innervate Cdh6 expressing visual targets. We screened a library of BAC transgenic mice (Gong et al., 2003) and found that Cdh3-GFP mice selectively label the RGCs that innervate Cdh6 expressing targets (Figure 2 and see Figure S1 available online). We injected CTb-594 into both eyes of Cdh3-GFP mice (ages P0-P20) and then examined each of

select their targets in the brain, the adhesion molecule Cdh6 is selectively expressed by a subset of non-image-forming retinorecipient targets.

Cdh3-RGCs Include Several Different Types, All of Which Express Cdh6

The limited number of retinorecipient targets innervated by Cdh3-RGCs prompted us to investigate which RGC types express GFP in this mouse line. Cdh3-RGCs represent ~1% of

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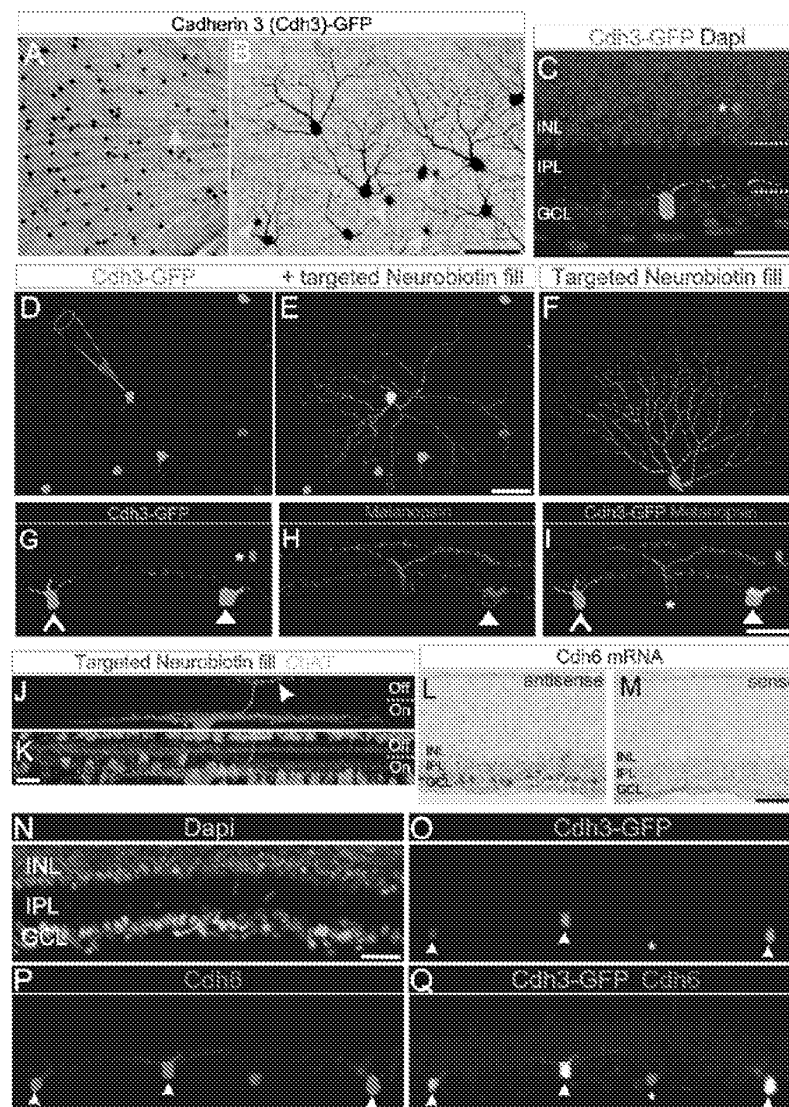


Figure 3. Cdh3-GFP and Cdh6 Expression in a Subset of Retinal Ganglion Cells

(A) GFP⁺ somas and axons in the ganglion cell layer (GCL) of a flat mounted Cdh3-GFP retina. Arrow: GFP⁺ axons. (B) GFP⁺ somas and dendrites of Cdh3-RGCs. GFP⁺ amacrine cell somas (arrowheads) are also seen deep to the plane of focus. Scale = 200 μm (A) and 50 μm (B). (C) Cdh3-RGC in a retinal section; the cell stratifies its dendrites proximal to the GCL, in the "On" sublamina of IPL. Dashed lines: IPL boundaries. Asterisk: a GFP⁺ amacrine. (D–F) Targeted intra-cellular injections with Neurobiotin (schematized in D; magenta label in E and F) reveal the morphology of individual Cdh3-RGCs. (D) Cdh3-RGCs without and (E) with injection. (E) A Cdh3-RGC with a symmetric, sparsely branched dendritic arbor. (F) A different filled Cdh3-RGC. This cell has a densely branched, asymmetric arbor (F). Scale = 50 μm (D–F). (G–I) A subset of Cdh3-RGCs express melanopsin. (G) Cdh3-RGCs (open and solid arrowheads) and an amacrine (asterisk). (H) Two RGCs and their dendrites expressing melanopsin (red). (I) Merge of (G) and (H). One of the Cdh3-RGCs expresses melanopsin (solid arrowhead) whereas the other does not (open arrowhead), a non-GFP⁺ melanopsin RGC is also present (asterisk). Scale = 50 μm. (J and K) Confocal z stack of a Neurobiotin-filled Cdh3-RGC whose dendrites stratify mainly in the On-sublamina of the IPL. The cell extends a secondary dendritic arbor into the Off-sublamina (arrowhead). (J) ChAT stained amacrine cell bodies and dendrites (blue). Scale = 10 μm (J and K). (L and M) Cdh6 mRNA is expressed in the GCL. (L) Cdh6 antisense. (M) Cdh6 sense control. Scale = 100 μm. (N–Q) Retinal section with (N) Dapi⁺ nuclei (blue), (O) Cdh3-RGCs, and (P) Cdh6 immunoreactive cells. (Q) Merge of (O) and (P). All Cdh3-RGCs express Cdh6 (arrowheads); but some Cdh6-immunopositive cells are not Cdh3-GFP⁺. Scale = 50 μm. See also Figure S3.

the total RGC population (mean Cdh3-RGCs per retina = 964.71 ± 57.62 GFP⁺; $n = 14$ retinas; 14 mice) (Jeon et al., 1998). Morphological analysis showed that approximately half (~47%; $n = 14/30$) of the Cdh3-RGCs had radial, sparse dendritic arbors (Figure 3E), whereas other Cdh3-RGCs (~53%; $n = 16/30$) had asymmetric, densely branching dendritic arbors (Figure 3F). Also, many Cdh3-RGCs had dendrites that stratified exclusively in the On sublamina of the inner retina, (e.g., Figure 3C) whereas other Cdh3-RGCs had dendrites stratifying in both the On and Off sublamina (Figures 3J and 3K). Approximately 10% of Cdh3-RGCs also expressed the photopigment melanopsin (Figures 3G–3I). Thus, Cdh3-RGCs are not a random sampling of RGC types, nor do they comprise a single RGC type. Rather, Cdh3-RGCs include a limited number of different RGC types.

We next wanted to determine whether Cdh3-RGCs also express Cdh6. We found that Cdh6 mRNA was expressed by a subset of cells in the early postnatal RGC layer (Figures 3L and 3M), which is in agreement with a previous report (Honjo et al., 2000). Immunostaining revealed that all Cdh3-RGCs also express Cdh6 protein (Figures 3N–3Q). However, not all Cdh6 immunoreactive cells were Cdh3-RGCs (Figures 3P and 3Q), suggesting that Cdh6-RGCs represent a broader population of RGCs. Consistent with this idea, we obtained brains from Cdh6-GFP transgenic mice in which GFP is localized to axon terminals by Gap43-EGFP fusion (Inoue et al., 2009). Cdh6-RGCs heavily target the vLGN, IGL, OPN, and mdPPN, just like Cdh3-RGCs. However, Cdh6-RGCs also projected to the medial terminal nucleus (MTN) and the SC and the MTN itself expressed Cdh6 mRNA (Figure S3). Thus, Cdh3-RGCs selectively innervate Cdh6 expressing retinorecipient targets and Cdh6-RGCs project to those same targets, as well as to additional Cdh6-expressing targets.



Cadherin-6 Promotes Axon-Target Recognition in a Non-Image-Forming Visual Circuit

The most widely held view of cadherin-mediated cell-cell interactions is a homophilic model whereby cells expressing specific cadherin family members preferentially bind to cells expressing the same cadherin or combination of cadherins (Takeichi, 2007). Thus, we hypothesized that Cdh6 is involved in matching the axons of Cdh3/6-RGCs to Cdh6-expressing targets. To address this, we mated Cdh3-GFP transgenic mice to Cdh6 mutant mice (Dahl et al., 2002) to generate Cdh3-GFP::Cdh6^{+/+} and Cdh3-GFP::Cdh6^{-/-} mice. In early postnatal Cdh3-GFP::Cdh6^{+/+} mice (n = 6 mice age P0/1; n = 6 mice age P5/6) and Cdh3-GFP::Cdh6^{-/-} mice (n = 5 mice age P0/1 and n = 3 mice age P6), the axons of Cdh3-RGCs target the rostral pretectum normally and form dense oval terminal zones corresponding to the OPN (Figures 4A and 4B). Similarly, the axons of Cdh3-RGCs terminate in two dense foci in the caudal pretectum of early postnatal Cdh3-GFP::Cdh6^{+/+} mice and Cdh6^{-/-} mice (Figures 4D and 4E), a pattern typical of early postnatal projections to the mdPPN (Figure S1). In contrast, early postnatal Cdh3-GFP mice that lacked Cdh6 (Cdh3-GFP::Cdh6^{-/-} mice) (n = 6 mice age P0/1; n = 5 mice age P6), had diminished axonal termination zones in the rostral pretectum (Figures 4C–4I). A similar lack of target recognition occurred in the caudal pretectum of Cdh3-GFP::Cdh6^{-/-} mice: the axons of Cdh3-RGCs were abnormally dispersed along the margin of the optic tract and they failed to aggregate into foci typical of the early postnatal mdPPN (Figure 4F). We occasionally found Cdh3-RGC axons projecting to nonvisual midbrain areas; something we essentially never encountered in wild-type mice of the same ages (Figure 4J). In most cases, however, the Cdh3-RGC axons that failed to terminate in the OPN and mdPPN projected through and past these targets to form ectopic terminations in the more distal retinorecipient area, the SC (Figures 4G–4I). The ectopic SC terminations appeared in the deepest retinorecipient layer, the stratum opticum (asterisks in Figure 4I) or as terminal arbors in the overlying stratum griseum superficialis (Figure 4K). This stands in contrast to wild-type (Cdh6^{+/+}) mice where Cdh3-RGC axons are rarely observed in the SC and when they are found there, they are generally confined to the caudomedial portion of the target (Figure 4G; asterisk). Together, these data indicate that when Cdh6 is deleted, the axons of Cdh3-RGCs fail to innervate their correct targets.

One interpretation of the defective target-innervation in Cdh3-GFP::Cdh6^{-/-} mice is that it reflects a failure for Cdh3-RGCs to recognize their proper targets. We can rule out two alternative interpretations. First, the reduced OPN and mdPPN innervation did not result from reduced numbers of Cdh3-RGCs in the Cdh6 mutants because they were present in normal numbers compared to wild-type mice (Cdh6^{+/+}; mean = 964 GFP RGCs; SEM = 57; n = 14 mice; versus Cdh6^{-/-}; mean = 1020 GFP RGCs; SEM = 68; n = 4 mice) (p = 0.63, two-tailed student's t test). Cadherins have been shown to play a role in sorting of motor neuron pools within the spinal cord (Price et al., 2002) which raises a second alternative interpretation of the mutant phenotype: that the axon targeting defects result from malformation of retinorecipient targets. To address this, we stained the brains of Cdh3-GFP::Cdh6^{-/-} mice with antibodies to parvalbu-

min, which labels a population of mature OPN cells (Prichard et al., 2002). In both Cdh6^{+/+} and Cdh6^{-/-} mice, parvalbumin-positive neurons are arranged in a dense aggregate that delineates the basic architecture of the OPN (Figures 4L and 4M). The Cdh3-RGC axons in Cdh6^{+/+} mice formed terminal arborizations throughout the parvalbumin neuron aggregates (Figure 4L), whereas in Cdh6^{-/-} mice, Cdh3-RGC axons were found in only a limited, lateral portion of the parvalbumin aggregate (Figure 4M). Thus, removing Cdh6 does not disrupt the formation or positioning of OPN neurons, which argues that the defective targeting in Cdh6 mutants reflects a failure of specific RGCs to recognize and terminate in their proper targets.

We noted variation in the severity of axon targeting defects in Cdh6 mutants, especially at ages P20 and older. In early postnatal animals (P0–P6), 3 out of 11 Cdh3-GFP::Cdh6^{-/-} mice exhibited apparently normal Cdh3-RGC axon targeting. In the remaining 8 Cdh3-GFP::Cdh6^{-/-} mice, the reduction in Cdh3-RGC input to the OPN ranged from severe (n = 5) to moderate (n = 3). By P20, 3 out of 7 Cdh6^{-/-} mice had no apparent targeting defects, 2 out of seven had severe phenotypes and 2 had moderate phenotypes. Examples of the variation in target innervation defects in the OPN and mdPPN are shown in Figure S4. This variation suggests that other molecules can compensate for early targeting errors caused by removal of Cdh6. One candidate is Cdh3. We attempted to create Cdh3-GFP::Cdh3^{-/-} mice in order to visualize the axons of Cdh3-RGCs in the Cdh3 null background but unfortunately those efforts failed, likely because the Cdh3-GFP transgene and the Cdh3 null cassette are located near one another on the same chromosome. However, whole-eye retinofugal tracing of Cdh3^{-/-} mutant mice indicated that eye-specific targeting to the dLGN and SC was normal. Input to image-forming areas was also normal in Cdh6^{-/-} mutant mice, as assessed by whole-eye anterograde labeling (Figures 4N–4P).

DISCUSSION

Deciphering the molecular basis of neural circuit specificity is a longstanding goal of neurobiology. The hypothesis that cadherins generate precise connectivity in the nervous system was initially put forth by Takeichi and coworkers (Suzuki et al., 1997; Inoue et al., 1998). Loss-of-function data support that model in lower vertebrates and flies (Inoue and Sanes, 1997; Lee et al., 2001; Prakash et al., 2005). Previous studies showed that distinct components of mammalian sensory circuits can be defined by their expression of different cadherins (Suzuki et al., 1997; Hertel et al., 2006), but evidence that cadherins play a functional role in generating wiring specificity in the mammalian CNS has been lacking. Here, we showed that Cdh6 mediates axon-target matching in a specific non-image-forming visual circuit. These data provide some of the first evidence that a specific classical cadherin can promote wiring specificity in the mammalian visual system.

The axon targeting defects we observed in Cdh6 knockout mice raise important questions about the mechanisms by which cadherins impart specificity of neural connections. The simplest explanation is that Cdh6-expressing RGC axons adhere to Cdh6 expressing target neurons via homophilic interactions that occur at the level of the targets. The ability of cadherins to localize to



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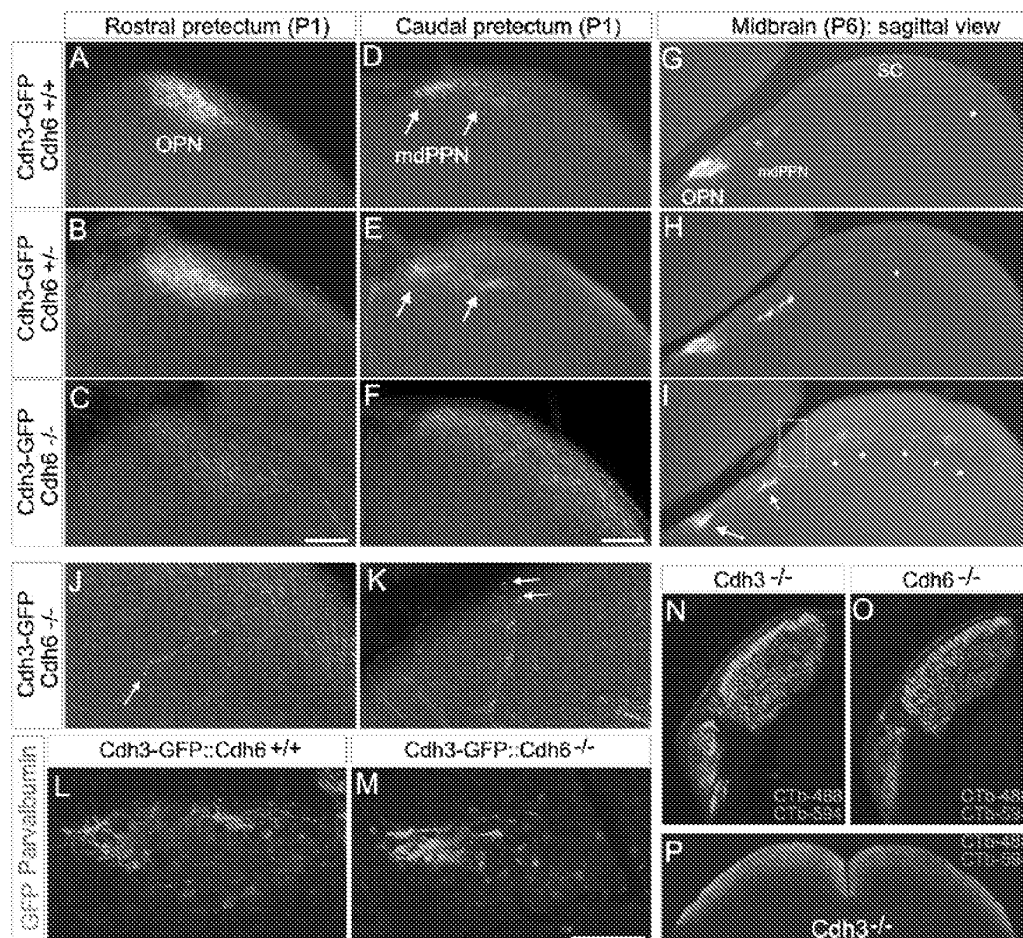


Figure 4. Cdh3-RGCs Display Target Recognition Errors in Cdh6 Knockout Mice

(A–F) Coronal view of Cdh3-RGC axon projections to the rostral (A–C) and caudal (D–F) pretectum of P1 mice. (A and D) Cdh3-GFP:Cdh6^{+/+}, (B and E) Cdh3-GFP:Cdh6^{-/-}, (C and F) Cdh3-GFP:Cdh6^{-/-} mice. In Cdh6^{+/+} and Cdh6^{-/-} mice, an oval termination zone characteristic of the OPN is apparent, whereas in Cdh6^{-/-} mice, Cdh3-RGC axons fail to terminate in the OPN region. In Cdh3-GFP:Cdh6^{+/+} mice (D) and Cdh3-GFP:Cdh6^{-/-} mice (E), two foci of GFP⁺ axon terminals are seen (arrows) whereas the Cdh3-GFP:Cdh6^{-/-} mice (F) lack these foci; instead Cdh3-RGC axons course through the caudal pretectum. Scale = 100 μ m (A–F). (G–I) Sagittal view of Cdh3-RGC axons in the pretectum and SC of P6 Cdh3-GFP:Cdh6^{+/+} (G), Cdh3-GFP:Cdh6^{-/-} (H), and Cdh3-GFP:Cdh6^{-/-} (I) mice. In Cdh3-GFP:Cdh6^{+/+} mice, Cdh3-RGC axons form a dense oval termination in the OPN and foci in the mdPPN; sparse axons are observed in the SC (asterisk). (H) In Cdh3-GFP:Cdh6^{-/-} mice, Cdh3-RGC axons terminate in OPN and in the mdPPN and SC. (I) In Cdh3::Cdh6^{-/-} mice, Cdh3-RGC axons display diminished terminal fields in the OPN region (larger arrow) and mdPPN (small arrow); asterisks: ectopic terminations in the SC. Scale = 500 μ m (G–I). (J and K) High-magnification view of Cdh3-RGC axons mistargeting in the OPN region (J) and in the rostral SC (K; from boxed region in I). Arrow in (J): a rare case of an axon seen projecting through the OPN region along the lateral-medial axis. Most misprojecting mutant axons travel through and past the OPN region along the rostral-caudal axis (as in C, F, and I, and see Figure S4). (L and M) Parvalbumin-expressing OPN neurons (magenta) of P20 Cdh6^{+/+} and P20 Cdh6^{-/-} mouse. Cdh3-RGC axons are found in the target cell region of both genotypes, but in Cdh6^{-/-} mice, they terminate in a lateral zone (M), rather than diffusely filling the entire aggregate of parvalbumin cells. Scale = 150 μ m. See main text and Figure S4 for quantitative details on phenotypic variation and examples of Cdh6 mutants. (N and O) Whole-eye anterograde labeling of RGC axons from the contralateral (CTb-488; green) and ipsilateral (CTb-594; magenta) eye in the visual thalamus (N and O) of a Cdh3^{-/-} and a Cdh6^{-/-} mouse. RGC axons are confined to their normal targets, the dLGN, IGL and vLGN (see Figures 1 and 3 for nuclei locations) and normal positioning of RGC axons is observed. (P) Eye-specific projections to the SC in a Cdh3^{-/-} mouse. Scale = 250 μ m.

synapses (Takeichi, 2007) supports that model. Alternatively, homophilic interactions among Cdh6 expressing RGCs may occur along the length of axons, en route to their targets. However, if the latter were the case, then we might expect to see defasciculation or axon growth deficiencies in Cdh6 mutants along the retinofugal pathway. We did not observe this; Cdh6

mutant axons arrived at their targets and indeed grew through and past them. They simply failed to terminate within those targets (e.g., Figures 4A–4I).

An alternative explanation is that the Cdh6 mutants phenotypes arise from heterophilic interactions among different cadherins. We did not examine Cdh6 binding specificity in this study,



but the expression of Cdh3 and Cdh6 in a single cohort of RGCs that innervate common targets (Figures 1–3), and the fact that Cdh2 is coexpressed with Cdh6 in those targets (Figures 11 and 1M), raises the possibility these cadherins generate target specificity by heterophilic interactions. The presence of multiple cadherins in the same neurons may also help explain why the Cdh6 null is not a fully penetrant phenotype: one cadherin may substitute in the others absence to reinforce proper axon-target connectivity. It is worth noting that age-dependent variability in phenotypes was also observed for kidney development in Cadherin-6 mutants (Mah et al., 2000).

Although not a fully penetrant phenotype, the absence of Cdh6 caused dramatic axon targeting defects in many cases, especially in early postnatal mice (Figures 4 and S4). The nature of those defects is informative toward understanding how cadherins impart specificity of connections: it was rare to observe mutant axons forming ectopic connections away from but in the vicinity of their normal targets. More often, the mutant axons traveled through their normal targets until they reached a different visual target, the SC. The fact that Cdh6 mutant axons grow through their targets but fail to stop and elaborate terminal arbors within them, supports the idea that removal of Cdh6 does not alter axon growth or guidance per se. Rather, Cdh6 appears necessary for axons to stop in the correct targets. The observation that misprojecting axons were able to invade the SC and form clustered terminations there (Figure 4I) also suggests that Cdh6 mutant axons are still capable of forming synapses. The location of those synapses is likely constrained by the guidance and activity dependent mechanisms that control afferent organization within that target, such as ephrins and spontaneous activity (Feldheim and O'Leary, 2010). Indeed, the retino-SC defects observed in Cdh6 mutants are reminiscent of the phenotypes observed in surgical "rewiring experiments" where RGC axons are forced into auditory nuclei. In those experiments, the misrouted RGC axons adopt terminal fields that are shaped by the local architecture and ephrin-based guidance systems they confront within the novel targets (Ellsworth et al., 2005).

Our data showing that an adhesion molecule is important for visual system wiring are consistent with previous reports showing that adhesion regulates RGC axon targeting at earlier points along the mammalian visual pathway (e.g., Williams et al., 2006) and with the abovementioned work in chicks and flies showing that N-Cadherin is important for lamina- and target-specificity (Inoue and Sanes, 1997; Lee et al., 2001; Prakash et al., 2005). Future studies that examine cadherin removal selectively in RGCs or in their targets, ought to shed further light on the mechanisms by which cadherins induce circuit specificity. In the meantime, our results provide evidence that cadherin mediated cell-cell adhesion is important for the establishment of functionally precise neural circuits in the mammalian visual system, by ensuring the appropriate sets of neurons in the eye connect to the appropriate sets of target nuclei in the brain.

EXPERIMENTAL PROCEDURES

Animals

Cadherin-3 BAC-EGFP (Cdh3-GFP) mice were obtained from MMRRC. Cdh6-GFP mice were generated as described in Inoue et al., (2009). Cdh6 knockout

mice were made as described in Dahl et al. (2002) and obtained from stocks at Jackson laboratories. All procedures were carried out according to protocols approved by animal care and use committees at UCSD, UCSC, Stanford, and NYU.

Immunostaining

Immunostaining for GFP was carried out as described in Huberman et al. (2008) using rabbit anti-GFP (Invitrogen; 1:1000), rabbit anti-melanopsin (AT5; 1:2000), goat anti-ChAT (Chemicon; 1:100), rabbit anti-Cdh6 (Dr. Gregory Dressler University of Michigan; 1:500) or mouse anti-parvalbumin (Chemicon; 1:2000) and Alexa-488, -594, or -647 secondary antibodies.

Labeling of Retinal Ganglion Cell Axons

Methods identical to those described in Huberman et al. (2008) were used. Briefly, a 5 μ l volume of 0.5% cholera-toxin beta (diluted in sterile saline) conjugated to Alexa-594 (Invitrogen) was injected into the right and left vitreous of Cdh3-GFP mice using a Hamilton syringe with 33 gauge needle. Twenty-four hours later, the mice were sacrificed, their brains removed and fixed for 24 hr in 4% PFA, then cryoprotected in 30% sucrose and sectioned at 35 μ m in either the coronal or sagittal plane.

In Situ Hybridization

Complementary DNAs for Cdh1 (nucleotides 1624–2193 of the mouse mRNA, NM_009864), Cdh2 (nucleotides 2253–2800, NM007664), Cdh3 (nucleotides 836–1356, NM_007665), Cdh4 (nucleotides 530–1273, NM_009867), Cdh5 (nucleotides 722–1293, NM_009868), Cdh6 (nucleotides 202–1229 NM_007666), Cdh7 (nucleotides 1029–1517, NM_172853), and Cdh8 (nucleotides 241–1481, NM_007667) were used to make antisense and sense digoxigenin-labeled RNA probes. In situ hybridization as previously described (Feldheim et al., 1998); protease K treatments were 1 mg/ml for 1 min.

Targeted Intracellular Filling of Ganglion Cells

Cdh3-RGCs were targeted and injected with Neurobiotin in live retinal explants then fixed and reacted with streptavidin-Cy3 (see Völgyi et al., 2009 for details).

Retinal Ganglion Cell Quantification

Cdh3-RGCs were counted from montaged high-resolution photomicrographs and then confirmed in the actual tissue specimen by comparison of the photo montages with the direct view of the retina on a Zeiss M1 microscope at 10–20 $\times$ magnification. Care was taken to only evaluate retinas where the entire whole mount was obtained by dissection. Student's t tests were used for statistical comparisons of RGC numbers between wild-type and mutant retinas.

SUPPLEMENTAL INFORMATION

Supplemental Information includes four figures and can be found with this article online at doi:10.1016/j.neuron.2011.07.006.

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Differential expression and function of cadherin-6 during renal epithelium development

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SUMMARY

The cadherin gene family encodes calcium-dependent adhesion molecules that promote homophilic interactions among cells. During embryogenesis, differential expression of cadherins can drive morphogenesis by stimulating cell aggregation, defining boundaries between groups of cells and promoting cell migration. In this report, the expression patterns of cadherins were examined by immunocytochemistry and *in situ* hybridization in the embryonic kidney, during the time when mesenchymal cells are phenotypically converted to epithelium and the pattern of the developing nephrons is established. At the time of mesenchymal induction, cadherin-11 is expressed in the mesenchyme but not in the ureteric bud epithelium, which expresses E-cadherin. The newly formed epithelium of the renal vesicle expresses E-cadherin near the ureteric bud tips and cadherin-6 more distally, suggesting that this primitive epithelium is already patterned with respect to

progenitor cell types. In the s-shaped body, the cadherin expression patterns reflect the developmental fate of each region. The proximal tubule progenitors express cadherin-6, the distal tubule cells express E-cadherin, whereas the glomeruli express P-cadherin. Ultimately, cadherin-6 is down-regulated whereas E-cadherin expression remains in most, if not all, of the tubular epithelium. Antibodies generated against the extracellular domain of cadherin-6 inhibit aggregation of induced mesenchyme and the formation of mesenchyme-derived epithelium but do not disrupt ureteric bud branching *in vitro*. These data suggest that cadherin-6 function is required for the early aggregation of induced mesenchymal cells and their subsequent conversion to epithelium.

Key words: Cadherin, Kidney development, Cell adhesion, Proximal tubule, Mouse

INTRODUCTION

The development of the renal epithelium from the metanephric mesenchyme and the ureteric bud requires inductive interactions, cellular proliferation and extensive tissue remodeling (Saxen, 1987; Ekblom, 1989). Much of the renal epithelium is derived from the metanephric mesenchyme, a group of morphologically distinct cells at the posterior end of the intermediate mesoderm. Upon induction by the ureteric bud epithelium, the metanephric mesenchymal cells aggregate and become polarized to generate a primitive, proliferating epithelial vesicle that ultimately generates the glomerular, proximal tubular and distal tubular epithelium. The analysis of mouse mutants and the use of *in vitro* organ culture methods have delineated many genes that are essential for these early inductive events and for the subsequent differentiation of the mesenchyme (Lechner and Dressler, 1997). Transcription factors that are essential during patterning and differentiation of the renal epithelium include Pax-2 (Torres et al., 1995), WT-

1 (Kreidberg et al., 1993), N-myc (Stanton et al., 1992) and Lim-1 (Shawlot and Behringer, 1995). In addition, the secreted signaling molecules Wnt-4 (Stark et al., 1995), BMP-7 (Dudley et al., 1995; Luo et al., 1995), GDNF (Pichel et al., 1996; Moore et al., 1996; Vega et al., 1996) and its receptor (Schuchardt et al., 1993) are also required for correct renal epithelial differentiation. How these developmental regulators exert their morphogenetic effects remains unclear. Ultimately, conversion of the metanephric mesenchyme must also be mediated, at least in part, by structural proteins that specify polarity, differential cell adhesion and cell migration.

Among the best characterized molecules that mediate cell-cell adhesion, the cadherins are calcium dependent, homophilic cell adhesion molecules that are implicated in pattern formation during development and signal transduction (Takeichi 1995; Gumbiner 1996). The cadherins generally contain five extracellular repeats, termed CAD domains, a transmembrane domain and a cytoplasmic tail that binds to the catenin family of cytoskeletal anchoring and signal transducing

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proteins (Ozawa et al., 1989, 1990). These CAD domains are responsible for the specificity of homophilic interactions between cells expressing the same cadherin. The prototype family member, E-cadherin, selectively interacts through the first extracellular CAD domain (Nose et al., 1990), the structure of which has recently been solved (Overduin et al., 1995). The ability of cells that express diverse cadherins to sort out and aggregate with cells expressing only like cadherins provides a molecular mechanism for tissue patterning, boundary formation and cell migration.

In this report, we have examined the expression of cadherins during the differentiation of the renal epithelium and the remodeling of the nephron in the mouse. In the process, we have also identified the mouse homologue of rat K-cadherin, or human cadherin-6, and generated antibodies specific for the cadherin-6 protein. Four cadherins were examined in a temporal sequence beginning with cadherin-11 in the uninduced mesenchyme, cadherin-6 in the mesenchymal aggregates, renal vesicle and proximal tubule progenitors, P-cadherin in the glomerular tuft and podocyte progenitors, and E-cadherin in the ureteric bud, collecting ducts and mature tubules. Differential expression of cadherin-6 and E-cadherin in the proximal and distal halves of the renal vesicle demonstrates, for the first time, that this primitive renal epithelium is already patterned with respect to its future derivatives. Furthermore, antibodies against the extracellular domain of cadherin-6 can interfere with the aggregation of induced mesenchymal cells and block the formation of mesenchyme-derived epithelium in kidney organ cultures.

MATERIALS AND METHODS

Screening of embryonic kidney cDNA libraries

A mouse embryonic day 17 kidney cDNA library was screened using degenerate PCR primers for cadherin cytoplasmic sequences as described by Suzuki et al. (1991) but with the addition of cloning nucleotides compatible with the CLONEAMP vector (Gibco BRL). The PCR conditions were: 5 cycles at 94°C, 80 seconds; 45°C, 2 minutes; 72°C, 2 minutes ramp; 72°C, 1 minutes; followed by 25 cycles at 94°C, 80 seconds; 55°C, 80 seconds; 72°C, 90 seconds. The PCR template DNA was isolated from a random primed, E17 mouse kidney cDNA library. A 50 µl PCR reaction contained 400 amol of each primer and 2.5 units Taq polymerase (Boehringer Mannheim), 0.5 mM dNTPs, and 1× buffer. The PCR products were ligated into the CLONEAMP vector and sequenced using the di-deoxy method (Sequenase, USB). The cadherin-6 PCR product was used to screen a mouse E17 kidney cDNA library. Several overlapping clones were identified that were greater than 97% identical to the rat K-cadherin sequence in the coding domain. In an independent screen, mouse cadherin-6 was isolated from an E9.5 cDNA library (gift of B. Hogan) under low-stringency conditions (Coffmann et al., 1990) using the entire coding region of *Xenopus* F-cadherin (Espeseth et al., 1995). The entire 789 amino acid cadherin-6 coding region was encompassed in a single clone. Sequence comparison revealed that both kidney-derived and whole-embryo-derived cDNAs encoded the same protein.

In situ hybridization

In situ hybridization with cadherin-11 was done essentially as described by Mackem and Mahon (1991) except ³³P-UTP was substituted for ³⁵S-UTP in the labeling reactions. The antisense cadherin-11 probes were derived from a cDNA clone that contained the entire coding region, as previously defined (Kimura et al., 1995;

Hoffman and Balling, 1995). The cadherin-11 probes used were a *Syl-EcoRV* fragment (nucleotides 1960-2535) and an *EcoRV-XbaI* fragment (2535-2890) that gave identical results upon hybridization to E11.5 and E15.5 sections. The antisense Pax-2 probe was a *BamHI-EcoRI* fragment described previously (Dressler et al., 1990). Labeling reactions contained 1 µg of linearized plasmid DNA and 100 µCi of ³³P-UTP (2000 Ci/mM, Amersham) and generally produced 3.5-4×10⁷ dpm of probe. Probes were diluted to 50,000 dpm/µl for hybridization and the slides were exposed for 28 days after emulsion coating.

Generation of cadherin-6 antibodies and Fab fragment purification

To produce a polyhistidine-tagged fusion protein in *E. coli*, a 1023 bp *BamHI* fragment, corresponding to amino acids 68-410, of the cadherin-6 cDNA 12A was inserted into the *BamHI* site of pRSET-B (In Vitrogen Inc.). Protein expression was induced with IPTG in the BL21 strain of *E. coli* and the protein was purified under denaturing conditions by nickel affinity chromatography. Purified protein was dialyzed stepwise into PBS. The cadherin-6 fusion protein was injected into rabbits (Babco Antibody Co.) and the animals were killed after the third boost. The antiserum was first purified by protein-A sepharose chromatography and then affinity purified using the cadherin-6 fusion protein fixed to an amino-link agarose column (Pierce). Fab fragments were prepared from affinity-purified α-cadherin-6 antibodies (cad6-Fab) or preimmune serum IgG (PI-Fab) by pepsin digestion (10 µg pepsin/mg Ab) in 100 mM NaOAc, pH 5.5, 50 mM cysteine, 1 mM EDTA for 2-4 hours at 37°C. Iodoacetamide was added to the reaction to a final concentration of 75 mM to stop the digestion. After 30 minutes at room temperature, the digested antibodies were dialyzed in 4 changes of 1 liter of PBS, for at least 8 hours per liter.

Cell aggregation

Mouse L cells (ATCC CCL-1) were transfected with a MMuLV retroviral vector (Rubenstein et al., 1984) containing the complete cadherin-6 coding region and transformants selected with 800 µg/ml G418 in DMEM/10% fetal calf serum. Individual colonies were picked, expanded and tested for cadherin-6 expression by western blotting. Two lines were identified as cadherin-6 positive (LK14 and LK17) and utilized for short-term aggregation assays as described by Nakagawa and Takeichi (1995). Monolayers of LK14 cells were treated with 0.01% trypsin and 1 mM CaCl₂ in Ca²⁺ and Mg²⁺ free 10 mM Hepes-buffered saline solution (HCFE) for 15 minutes at 37°C on a gyrotary shaker. The cells were pelleted and, after discarding the supernatant, 100 µl of 0.5% (w/v) soybean trypsin inhibitor in HCFE was added to the cell pellet. The cells were washed three times with ice-cold HCFE. Cells were then resuspended at 2-5×10⁵ cells/ml in HCFE using Pasteur pipette to get single-cell suspension. Aggregation assay was performed in 24-well plate pre-coated with 1% BSA in HCFE. 500 µl of cell suspension in HCFE, with 1 mM CaCl₂, with 1 mM CaCl₂ and 17 µg/ml PI-Fab, or with 1 mM CaCl₂ and 17 µg/ml cad6-Fab was added to each well. The plate was rotated on a gyrotary shaker at about 80 revs/minute at 37°C. After 2 hours of incubation, cells in each well were photographed.

Immunostaining

FVB/N inbred mice were mated and the day of the vaginal plug was designated E0. Embryos were dissected free of extraembryonic tissue and frozen on dry ice. Commercial reagents utilized were: anti-Laminin Ab (Sigma #L-9393); anti-E-cadherin Ab (Sigma #U-3254); anti-WT1 Ab (Santa Cruz #sc-192); anti-Pan Cytokeratin Ab (Sigma #C-9687); anti-P-cadherin Ab (Zymed Labs. #13-2000); FITC-conjugated Lotus Tetragonobulus (LTA, Sigma #L-9254). Immunostaining was performed as described by Harlow and Lane (1988). Cryostat sections of fresh frozen embryos were cut at 8 µm, collected on gelatinized slides and air dried for 30-60 minutes. Sections

were fixed in 4% paraformaldehyde/PBS for 10 minutes, then washed twice in PBS/0.05% Tween-20 for 5 minutes. Antibody dilutions were 1:400 for E-cadherin Ab, 2 µg/ml for α -cadherin-6 Ab, 1:100 for WT1 Ab, 1:20 for P-cadherin Ab and 10 µg/ml of α -Pax-2 IgG in 2% goat serum in PBS. Fluorescein-conjugated Lotus Tetragonolobus was diluted 1:20. Slides were incubated at room temperature with 20 µl of antibody in a humid chamber and then washed twice in PBS/0.1% Tween-20 (PBST). The second antibodies were diluted 1:400 for the Texas Red-conjugated anti-rabbit (Molecular Probes) and 1:20 for the FITC-conjugated anti-rat (Sigma) in 2% goat serum in PBS. After a 30 minutes incubation, slides were washed twice in PBST and covered with gelvatol. Control sections were incubated with a preimmune rabbit IgG fraction and both second antibodies. No specific staining could be detected with the preimmune IgG from rabbits immunized with the cadherin-6 antigen (data not shown).

For whole-mount antibody staining of organ cultures, kidney rudiments were fixed in 100% methanol for 10 minutes and washed twice in PBST for 20 minutes. Tissues were incubated for 4-5 hours with primary antibodies at 4°C and washed overnight in PBST at 4°C. Incubation with secondary antibodies was 4-5 hours at 4°C and multiple washes were performed with a large volume PBST overnight at 4°C. Whole-mount tissues were placed under coverslips in gelvatol for microscopy.

Organ culture

Kidneys were dissected from E11.5 littermates in Ham's F12 media and cultured on Transwell filter inserts (Costar) with 3.0 µm pore sizes. Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10% fetal calf serum and penicillin/streptomycin was used for all experiments. For the antibody blocking experiments, the kidney rudiments were incubated for 1 hour in DMEM with 30 µg/ml of PI-Fab or cad6-Fab before placing on the filters and then cultured for an additional 48 hours in the presence of antibodies.

RESULTS

Cadherin genes in the embryonic kidney

In order to more precisely define the different cadherins that are expressed in the developing kidney, we screened an E17 kidney cDNA library using degenerate PCR primers designed to amplify the conserved cadherin cytoplasmic domain. The PCR products were cloned into a plasmid vector and 21 independent clones were identified. Sequence analysis revealed that three clones were identical to mouse uvomorulin, or E-cadherin (Nagafuchi et al., 1987; Ringwald et al., 1987), three clones corresponded to P-cadherin (Nose et al., 1987), six clones were identical to rat K-cadherin (Xiang et al., 1994) or mouse cadherin-6 (Inoue et al., 1997), and nine clones were identical to cadherin-11 (Kimura et al., 1995; Hoffman and Balling, 1995). The mouse cadherin-6 PCR fragment was used to isolate the entire coding region from an E17 embryonic kidney cDNA library. Concurrently, a mouse E9.5 cDNA library was screened with a *Xenopus* F-cadherin probe under low-stringency conditions. A single 3.3 kb clone was identified (c12A) that contained the entire cadherin-6 open reading frame. The sequence was determined to be identical with that reported by Inoue et al. (1997).

Expression of cadherins in the developing kidney

In order to determine how the cadherins might function in the differentiation of the renal epithelium, the temporal and spatial expression patterns of the different cadherins were analyzed by in situ hybridization or immunocytochemistry in the developing kidney. Cadherin-11 is expressed primarily in mesenchymal cells throughout the embryo and has been described in detail (Kimura et al., 1995; Hoffman and Balling, 1995). However, in the induced mesenchyme of the E11.5-day kidney, cadherin-11 is expressed more strongly and overlaps with the Pax-2-positive mesenchymal cells at this stage (Fig. 1A-D). Cadherin-11 is not expressed in any epithelial structures, including the ureteric bud (Fig. 1C) and the developing renal tubules (Fig. 1E) that are derived from the cadherin-11-expressing mesenchymal cells. Cadherin-11 continues to be expressed in the interstitial mesenchymal cells of the kidney at later stages (Fig. 1E). Although high levels are initially detected in the induced mesenchymal cells, cadherin-11 is down-regulated as these induced cells become polarized and form the undifferentiated epithelium of the renal vesicle.

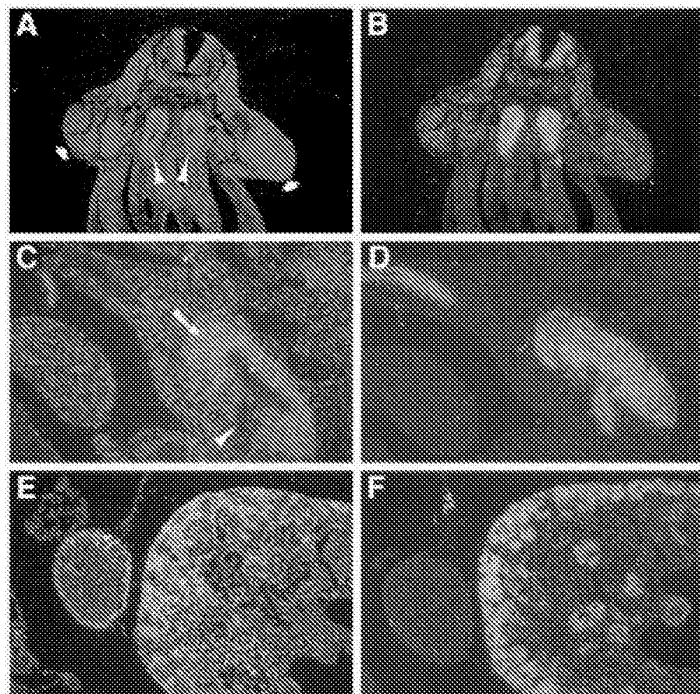


Fig. 1. Expression of cadherin-11 and Pax-2 mRNAs in the developing kidney. In situ hybridization using probes specific for cadherin-11 (A,C,E) and Pax-2 (B,D,F) are shown in serial sections through the kidney at various embryonic stages. (A,B) Transverse section through an E11.5 embryo showing cadherin-11 in the metanephros (arrowhead), limb buds (arrow) and other mesenchymal cell types. Pax-2 is restricted to the metanephros and the neural tube. (C,D) Sagittal sections through an E11.5 kidney. Note, cadherin-11 is only in the mesenchymal cells (arrow) and not in the ureteric bud epithelium (arrowhead), whereas Pax-2 is expressed in both cell types. (E,F) Sagittal sections through an E14.5 kidney showing persistent cadherin-11 expression in the interstitial mesenchymal cells but not in the mesenchymal aggregates along the periphery, where Pax-2 expression is still high. Micrographs were taken with a Nikon Microphot-FXA and 35 mm film with a total magnifications of 12 $\times$ (A,B), 64 $\times$ (C,D), 32 $\times$ (E,F).

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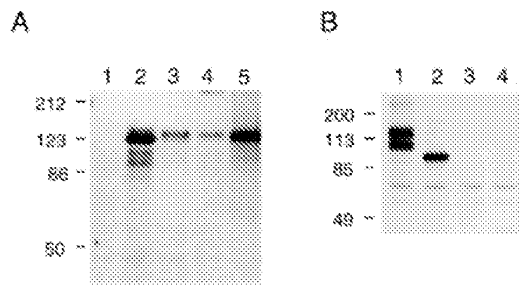
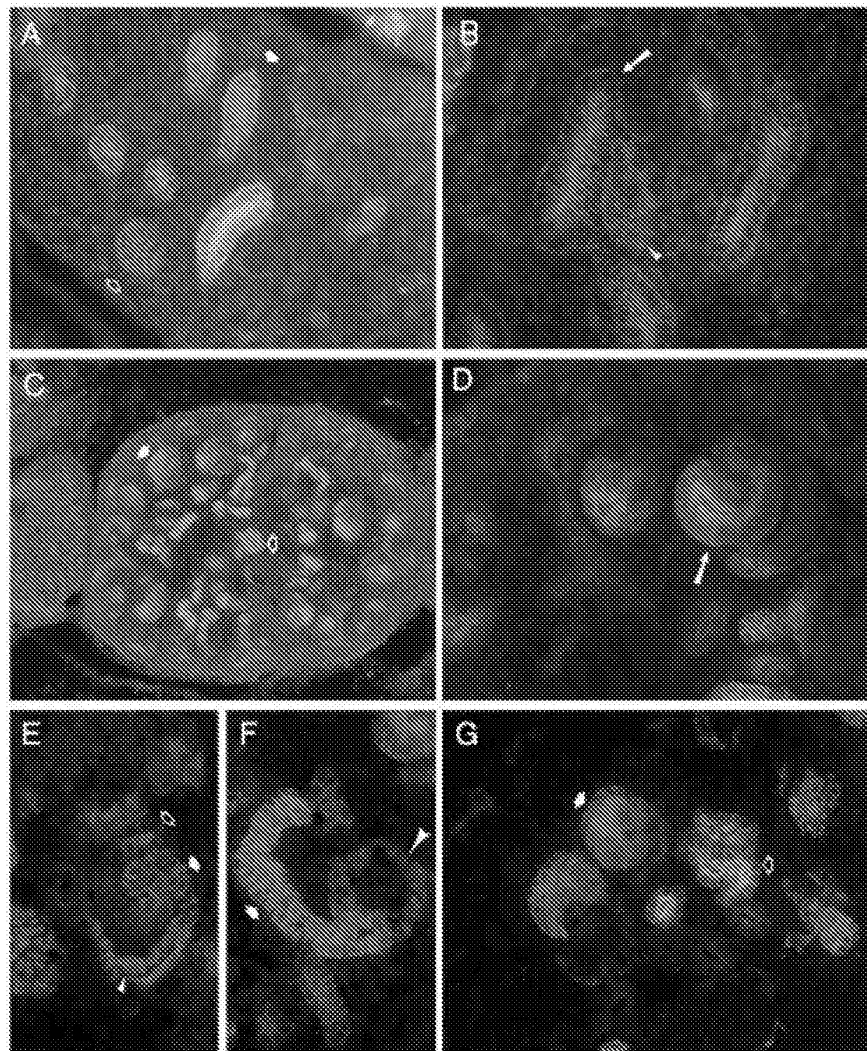


Fig. 2. Specificity of α -cadherin-6 antibodies. Western blots probed with affinity-purified α -cadherin-6 antibodies. (A) Lane 1, control vector transfected LTK- cells; lane 2, CMV-cadherin-6 transfected LTK- cells; lane 3, renal cell carcinoma line UCK111; lane 4, renal cell carcinoma line UCK117; lane 5, E17.5 kidney protein lysate. (B) NIH 3T3 transiently transfected cells. Lane 1, CMV-cadherin-6; lane 2, CMV- Δ C-cadherin-6; lane 3, CMV-cadherin-11; lane 4, CMV control vector.

In order to analyze the protein product of the cadherin-6 gene, antibodies were generated against a bacterial protein that contained amino acids 68 to 410 fused to a polyhistidine tag. The polyclonal rabbit antibodies were purified first over a protein-A agarose column and subsequently by affinity chromatography to the fusion protein. Specificity of the purified α -cadherin-6 antibodies is demonstrated by western blotting (Fig. 2). The α -cadherin-6 recognizes a single protein in extracts of embryonic kidney that migrates at approximately $120 \times 10^3 M_r$. A similar species is detected in human renal carcinoma cell extracts, consistent with the reported expression of cadherin-6 in renal cell carcinoma (Xiang et al., 1994). A similar size protein is detected in LTK- cells transfected with a CMV-cadherin-6 expression plasmid, but not in vector only transfected cells. Since the predicted molecular mass of the mature peptide would be approximately $81 \times 10^3 M_r$, the increase in apparent molecular mass is probably due to post-translational modifications such as N-linked glycosylation.

Because cadherin-11 is the most closely related to cadherin-

Fig. 3. Immunocytochemistry of E-cadherin and cadherin-6 in the embryonic kidney. (A) E12.5 mesonephros showing E-cadherin (green) along the dorsal side (open arrow) where the nephric duct is located and cadherin-6 (red) at the ventral end (solid arrow) of the tubules. Both E and cadherin-6 are expressed in a transition zone (yellow) located between the nephric duct and the mesonephric glomeruli. (B) E12.5-day metanephros showing E-cadherin (green) in the ureteric bud (arrow) and cadherin-6 (red) in the renal vesicle (arrowhead). Note E-cadherin expression in renal vesicle cells directly abutting the ureteric bud termini. (C) E15.5 metanephros showing E-cadherin (green) and cadherin-6 (red). Cadherin-6 is still prominent in the s-shaped bodies (solid arrow) but more mature tubules express both K and E-cadherin (open arrow). (D) E15.5 comma-shaped body (arrow) expressing cadherin-6 (red) at the proximal end and E-cadherin (green) at the distal end, adjacent to the ureteric bud. (E) E15.5 S-shaped body expressing E-cadherin (green) at the distal end, cadherin-6 (red, cell surface) more proximally, and WT1 (red, nuclear) in the podocyte progenitors of the glomerular cleft. (F) E15.5 Glomerulus expressing WT1 (red, nuclear) and proximal tubule expressing cadherin-6 (red, cell surface). Note the absence of cadherin-6 in the glomerular epithelium. (G) E15.5 metanephros expressing cadherin-6 (red) in the comma- and s-shaped bodies and the developing proximal tubules which also stain with the LTA (green). Total magnification is $25\times$ (C) and $100\times$ (A,B,D-G).



6, being 61% identical across the extracellular domain used for immunization, additional specificity controls were done to exclude potential cross reactivity of α -cadherin-6 to cadherin-11 (Fig. 2B). The α -cadherin-6 antibodies recognized the major $120 \times 10^3 M_r$ form and several smaller bands in cadherin-6 transiently transfected 3T3 cells. Given the high levels of expression observed upon transient transfection, it is likely that the smaller bands represent unmodified cadherin-6. Cells transfected with a vector expressing a truncated form of cadherin-6 (ΔC), minus the cytoplasmic domain, exhibited a single band at approximately $90 \times 10^3 M_r$. Cells transfected with a cadherin-11 expression vector did not show any proteins detectable with α -cadherin-6 above control vector transfected cells. Cross reactivity to E-cadherin is unlikely given only 36% homology in the extracellular domain between E-cadherin and cadherin-6.

Antibodies against cadherin-6, E-cadherin and Pax-2 were used to stain embryonic kidneys at various stages of development. The pattern of Pax-2 expression has been described in detail (Dressler et al., 1990; Dressler and Douglass, 1992; Ryan et al., 1995). At E12.5, the nephric duct extends from anterior to posterior within the more dorsal intermediate mesoderm and expresses E-cadherin (Fig. 3A). Along the more anterior nephric duct, the mesonephric tubules are still present as a linear array in the sagittal plane. These mesonephric tubules are transient embryonic structures that consist of a glomerular capsule, near the dorsal aorta, and a convoluted tubule emptying into the nephric duct. These tubules are patterned along the dorsal-ventral axis with respect to cadherin expression (Fig. 3A). The mesonephric tubules express cadherin-6 in the most ventral region, near the glomerular capsule. As the tubules extend dorsally towards the nephric duct, there is a region of overlap where both cadherin-6 and E-cadherin are expressed. Finally, as the tubules reach the nephric duct, only E-cadherin remains.

At the posterior end of the E12.5 nephric duct, the ureteric bud has grown out and induced the metanephric mesenchyme to generate the definitive kidney or metanephros. Pax-2 expression is prevalent in both ureteric bud epithelium, induced metanephric mesenchyme and the epithelial derivatives of the mesenchyme (Dressler et al., 1990; Dressler and Douglass, 1992; Ryan et al., 1995). E-cadherin is prevalent in the ureteric bud and its branches. The expression of cadherin-6

can first be detected in the mesenchymal aggregates that will form the epithelium of the renal vesicle at E12.5 (Fig. 3B). Initially, cadherin-6 is detected on the apical side of the newly formed epithelium and subsequently demarcates the entire cell surface. Cadherin-6 expression is highest in those cells furthest from the ureteric bud termini, whereas some cells adjacent to the ureteric bud termini express E-cadherin. The expression of cadherins in the E15.5 kidney is complex and reflects the ongoing morphological changes in the developing renal epithelium. Cadherin-6 is still prevalent in the renal vesicles at the periphery of the kidney, with E-cadherin throughout the ureteric bud epithelium and the major collecting ducts derived from the ureter (Fig. 3C). In the comma-shaped bodies (Fig. 3D), cadherin-6 is strong in the future proximal end of the body, furthest from the ureteric bud, while E-cadherin is expressed in the more distal part, nearest to the ureter. To minimize confusion, we will use proximal and distal with respect to the future position of the glomerulus, as is the case with the mature nephron. Only a small region of overlap can be detected between cadherin-6 and E-cadherin at this time. As the glomerular cleft is formed at the proximal end of the comma-shaped body to generate the s-shaped body (Fig. 3E,F), cadherin-6 is down-regulated in the epithelium of the glomerular cleft, the prospective podocytes. The podocyte progenitors can be stained for the nuclear antigen WT1 (Fig. 3E,F) and clearly show little cadherin-6 on the cell surface. These WT1-expressing podocyte progenitor cells have also down-regulated the Pax-2 gene (Ryan et al., 1995). Cadherin-

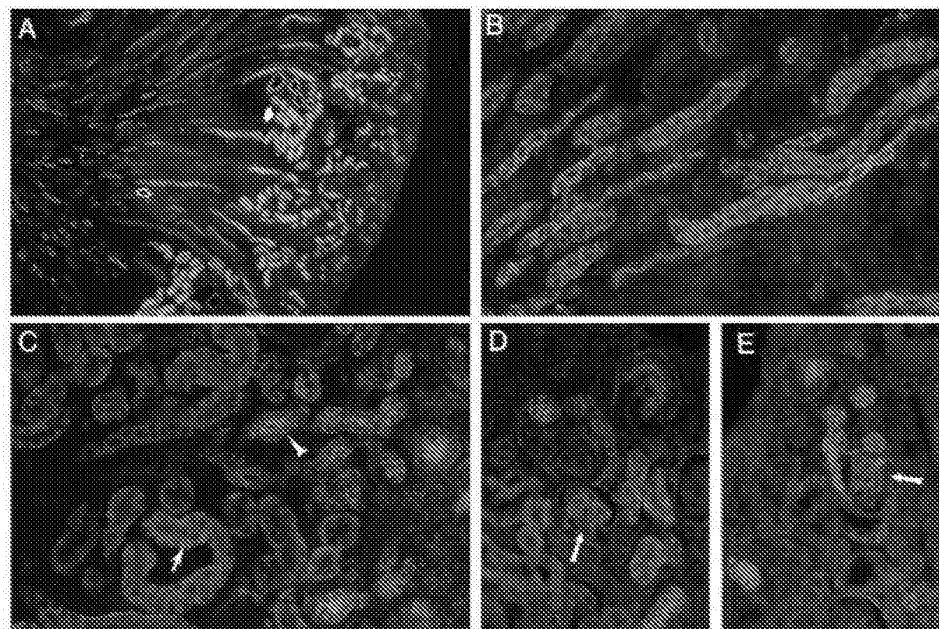


Fig. 4. Immunostaining of cadherins in the newborn kidney. (A) Cadherin-6 (red) persists in the nephrogenic zone along the periphery and in the medullary zone (open arrow). Mature proximal tubules stain with LTA (green) and have down-regulated cadherin-6 (solid arrow). (B) Newborn renal medulla showing E-cadherin (green) in the collecting ducts and cadherin-6 (red) in the ascending and descending limbs of Henle's loop. (C) Newborn renal cortex exhibits less cadherin-6 (red) in maturing proximal tubules with punctate E-cadherin (green) now detectable in most epithelial cells. (D,E) Newly formed glomeruli express P-cadherin (green) in cells of Bowmans capsule and cadherin-6 in the proximal tubules exiting the glomerulus. Total magnification is $25\times$ (A) and $100\times$ (B-E).

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6 is still strong in the prospective proximal tubule cells of the s-shaped body with E-cadherin marking the prospective distal tubule cells nearest the developing collecting ducts. As in the developing mesonephros, there is a transition zone within the s-shaped body where E-cadherin and cadherin-6 are co-expressed.

The cadherin-6-expressing cells are primarily destined to become proximal tubule epithelium as indicated by the expression of the lectin-binding sites for Lotus Tetragonolobus (LTA, Laitinen et al., 1987). In the E15.5 kidney, fluorescein-conjugated LTA stained only cadherin-6-expressing cells. These double-labeled cells were generally more mature than cadherin-6-expressing cells located along the peripheral, nephrogenic zone, which did not express the LTA-binding site (Fig. 4A). In the newborn kidney, cadherin-6 expression is strong in two particular regions, in the nephrogenic zone where the mesenchymal-to-epithelial transition is still ongoing and in the medullary zone where proximal tubules are extending radially inward to form the descending and ascending loops of Henle (Fig. 4B). The more mature proximal tubules are easily marked with LTA and have down-regulated cadherin-6 expression. E-cadherin is now detected in those cells that had expressed cadherin-6 at earlier times. E-cadherin is also prevalent in the collecting ducts of the medullary zone (Fig. 4C) but does not overlap with cadherin-6 in this region. In the glomerular epithelium, the expression of P-cadherin can now be detected in the podocyte epithelium. The expression of P-cadherin is similar to previously reported observations (Tassin et al., 1994).

Antibodies against cadherin-6 inhibit aggregation and formation of tubules

Given the expression of cadherin-6 in the mesenchymal aggregates that form at the tip of the ureteric bud, we hypothesized that cadherin-6 may mediate the aggregation and polarization of these mesenchymal cells to generate proximal tubule epithelium and perhaps other parts of the nephron. In order to test this hypothesis, Fab fragments were made by papain digestion of the affinity-purified anti-cadherin-6 polyclonal antibodies (cad6-Fab) and from the pre-immune IgG fraction (PI-Fab). The antibodies were tested for their ability to inhibit cadherin-6-mediated aggregation using transfected L cells. LK14 cells stably expressing cadherin-6 were generated by retroviral-mediated gene transfer and utilized in short-term aggregation assays (Fig. 5). Calcium-dependent cellular aggregates could be observed with LK14 cells whereas the parental L cell line showed no evidence of aggregation (data not shown). Aggregation of LK14 cells could be inhibited by the addition of cad6-Fab but not with the control PI-Fab fraction (Fig. 5). Thus, cadherin-6-dependent homophilic adhesion could be abrogated with the polyclonal antibodies against the extracellular domain.

In order to study the effects of cadherin-6 inhibition during renal development, the antibodies were added to the medium of E11 kidney rudiments and the kidneys cultured for 48 and 96 hours. The degree of development of cultured kidneys was examined by immunohistochemistry using antibodies against E-

cadherin, cytokeratins, cadherin-6, laminin, Pax-2 and WT1 (Fig. 6). Three separate experiments utilizing at least 6 E11 kidney rudiments for each culture condition were performed. In all cultures examined, there was no significant difference between kidneys cultured in DMEM alone and DMEM supplemented with 30 µg/ml of PI-Fab. Ureteric bud branching occurred repeatedly as detected by both cytokeratin and E-cadherin staining (Fig. 6A,C). Mesenchyme-derived epithelium was easily distinguishable as Pax-2-positive, comma- or s-shaped tubules adjacent to the ureteric bud branches (Fig. 6A). The mesenchyme-derived epithelium did not stain with the cytokeratin antibodies, but did have a laminin-containing basement membrane (Fig. 6E) and expressed either E-cadherin or cadherin-6 (Fig. 6C). Similar to the situation *in vivo*, mesenchyme-derived tubules expressed E-cadherin near the ureteric bud and cadherin-6 further from the bud. Strikingly, cadherin-6 was first visible on the mesenchymal cells at the extreme tip of the bud, on the prospective apical side of the cells undergoing polarization (Fig. 6C arrowhead).

In contrast, kidney rudiments cultured in the presence of 30 µg/ml cad6-Fab exhibited significant differences in the formation of mesenchyme-derived epithelium. The cad6-Fab had no effect on ureteric bud branching. However, the ability of mesenchymal cells to condense near the ureteric bud tips and generate epithelium was severely compromised. Activation of Pax-2 is an early marker of mesenchymal induction and appeared undisturbed by any antibody treatment (Fig. 6B). However, in the cad6-Fab-treated kidneys, Pax-2-expressing

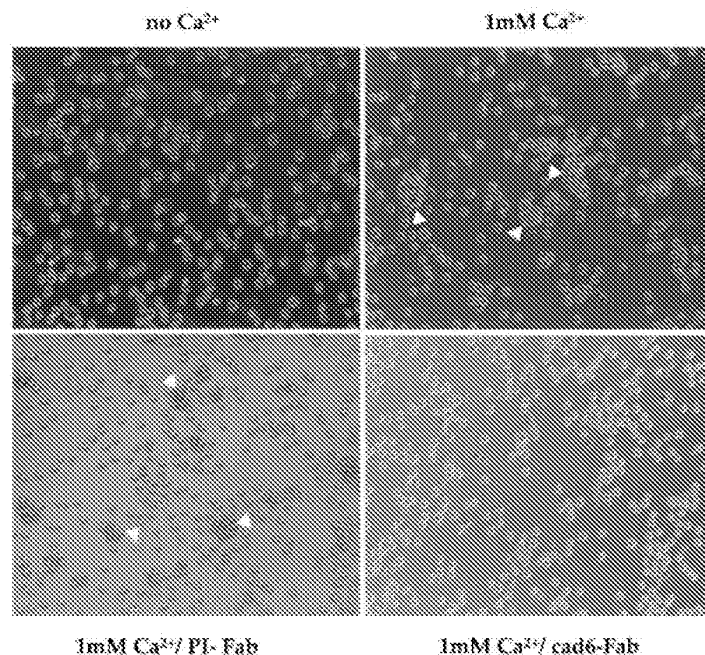


Fig. 5. Inhibition of cadherin-6-dependent aggregation by cad6-Fab. LK14 cells expressing cadherin-6 were trypsinized and allowed to reaggregate in the presence or absence of calcium. Note the formation of cellular aggregates (arrowheads). Cad6-Fab but not control PI-Fab antibodies inhibit the calcium-dependent aggregation.

cells at the ureteric bud tips were very loosely associated and many Pax-2-positive cells were localized throughout the rudiment. In contrast, controls showed tightly associated Pax-2-positive cells separated by Pax-2-negative, presumably interstitial, mesenchyme (Fig. 6A). The formation of comma- and s-shaped bodies was severely inhibited in cad6-Fab-treated cultures as clearly indicated by the lack of polarized epithelium with a laminin-staining basement membrane (Fig. 6F). Almost all of the epithelium in the cad6-Fab-treated cultures was derived from the ureteric bud as indicated by cytokeratin (Fig. 6F) and E-cadherin staining (Fig. 6D). The whole cadherin-6 antibodies detected very little cadherin-6 reactivity in the cad6-Fab-treated samples (Fig. 6D). Notably, those cells that were cadherin-6 positive were localized in the interior of the organ culture and did appear to be epithelial. After 96 hours, the presence of glomerular structures were detected using an antibody against WT1, which is highly expressed in the podocyte precursor cells of the s-shaped body and in mature glomeruli (Ryan et al., 1995). Kidney rudiments cultured with cad6-Fab exhibited few, if any normal glomerular structures (Fig. 6H), whereas controls showed round clusters of WT1-positive cells (Fig. 6G) indicative of glomerular podocyte development.

DISCUSSION

The development of the nephron from the two primary progenitor cell types, the ureteric bud epithelium and the metanephric mesenchyme, requires a phenotypic conversion of mesenchymal cells to an epithelial phenotype and a complex series of morphogenetic events that pattern the epithelium along the proximal-distal axis. The ability to concentrate urine, sense and regulate osmolarity, and continuously feed back information to the glomerular apparatus requires a precise three-dimensional architecture in the nephron that is intimately linked to proper function. How this architecture is specified during development must be regulated not only by specific transcription factors but also by proteins that provide the motor forces underlying cell movement and tissue remodeling.

The expression pattern of cellular adhesion molecules can demarcate the developing renal epithelium into functionally and morphologically distinct regions as the mesenchyme aggregates, forms the renal vesicle and ultimately

generates a mature nephron. The data presented in this report are summarized schematically in Fig. 7. The *cadherin-11* gene is expressed in a large variety of mesenchymal cell types

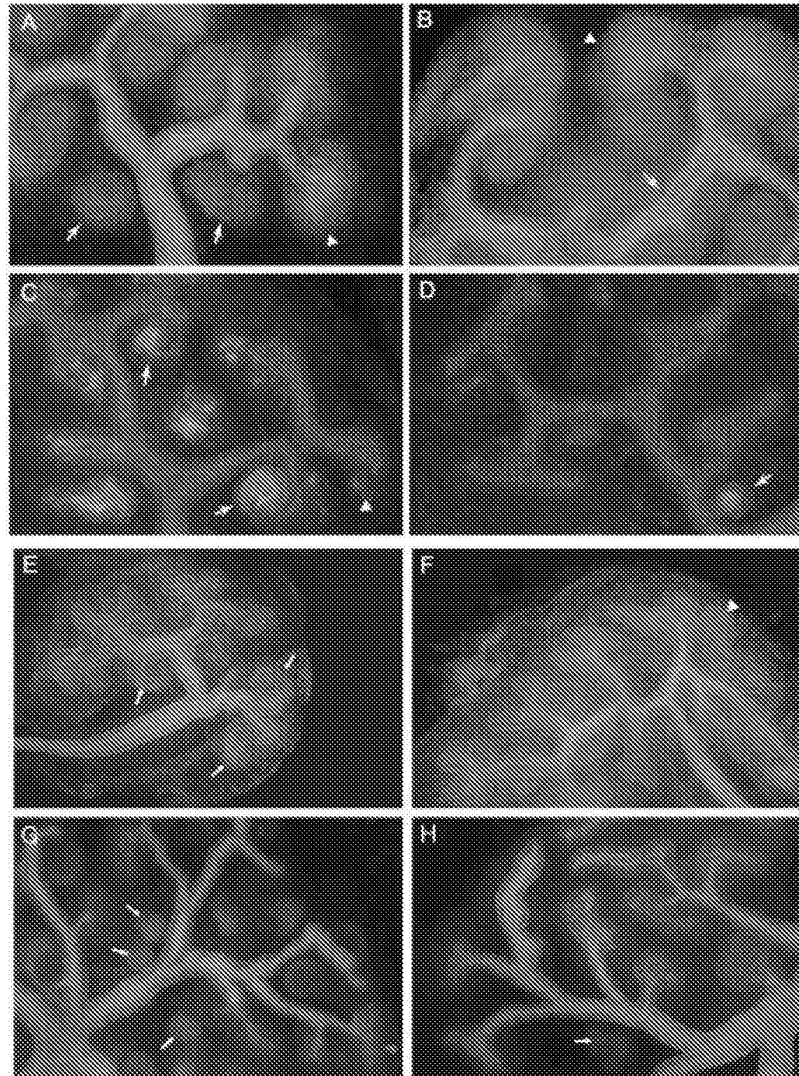
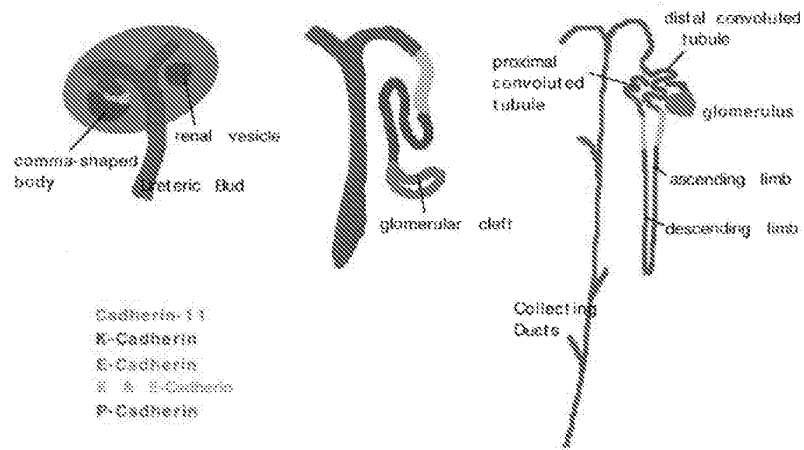


Fig. 6. Inhibition of mesenchyme-derived tubulogenesis by α -cadherin-6 antibodies. E11.5 metanephric rudiments cultured for 48 hours with PI-Fab (A,C,E) or cad6-Fab (B,D,F) and stained whole mount with specific antibodies. (A,B) Staining with Pax-2 (red) and cytokeratin (green) shows loosely associated induced mesenchyme (B, arrowhead) at the tips of ureteric bud in cad6-Fab cultures. Controls exhibit tight association (A, arrowhead) and comma shaped bodies (A, arrows). Also note Pax-2-positive cells in B (arrow) that line the more mature ureteric bud epithelium, this is never observed in controls. (C,D) Staining for cadherin-6 (red) and E-cadherin (green) reveals well-developed comma- and s-shaped bodies in controls (C, arrows) and little cadherin-6 protein in cad6-Fab-treated cultures; the occasional positive structure (D, arrow) appears epithelial. (E,F) Staining for laminin (red) and cytokeratin (green) reveals well-developed basement membranes around comma- and s-shaped bodies in controls (E, arrows). In cad6-Fab-treated cultures, basement membrane staining is clear in ureteric bud epithelium, positive for cytokeratin, but diffuse staining in cells adjacent to the ureteric bud tips. Only occasional, poorly developed structures show characteristic basement membrane formation in mesenchyme-derived epithelium (F, arrowhead). (G,H) Staining for WT1 (red) and cytokeratin (green) after 4 days shows well-developed glomerular structures (G, arrows) in controls and few, poorly developed glomeruli in cad6-Fab-treated cultures (H, arrow).

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Fig. 7. Summary of cadherin expression during nephron development. Induced mesenchymal cells repress cadherin-11 and activate cadherin-6; mesenchymal cells closest to the ureteric bud activate E-cadherin. The s-shaped body is patterned with respect to cadherin expression such that glomerular precursors express P-cadherin, proximal tubule precursors express cadherin-6 and more distal tubule precursors express E-cadherin. In newborns, cadherin-6 becomes down-regulated in maturing proximal tubules but can still be seen in Henle's loop.



throughout embryogenesis, with strikingly high levels in the metanephric mesenchyme, relative to surrounding mesoderm. The cells of the uninduced metanephric mesenchyme can be distinguished morphologically from surrounding tissue because they are more tightly associated. Whether this is due to increased levels of cadherin-11 is unclear. After induction occurs at E11, cadherin-11 expression levels do not appear to be higher in the cells directly adjacent to the ureteric bud tips where the aggregates first form and levels are quickly down-regulated as evidence of epithelial transformation is apparent. Yet, cadherin-11 could still function in the earliest adhesion response to induction, perhaps by the controlled activation of other factors that interact with the intracellular domain such as the catenin family of proteins (Ozawa et al., 1989, 1990) thus providing a rapid response to the inductive signals.

Cadherin-6 is first detected in the mesenchymal aggregates and in the renal vesicle before a polarized epithelium, complete with basement membrane, is generated. Particularly in organ culture, where the timing of events may be slower, cadherin-6 is first detected as a patchy strip around the extreme ureter bud tips, suggesting that its expression is first localized to one side of the mesenchymal cell and precedes the formation of the polarized epithelium. At E12.5, the renal vesicle already appears patterned with respect to cadherin expression. The cells adjacent to the ureteric bud express primarily E-cadherin whereas the more distal cells, relative to the ureteric bud, express cadherin-6. Thus, the epithelium of the renal vesicle is not a homogenous population of primitive epithelial cells. The proximity to the ureteric bud and its inductive signals may determine the type of cadherin expressed in the vesicle and the ultimate fate of the epithelium. Alternatively, the expression of E-cadherin may in fact reflect the origin of the cells in that part of the renal vesicle, as recent evidence points to a population of cells that can delaminate from the ureteric bud and contribute to the tubular epithelium that was previously thought to derive only from the mesenchyme (Qiao et al., 1995). At the s-shaped body stage, differential cadherin expression clearly demarcates the precursor cells of distal tubules as E-cadherin positive, the proximal tubules that express cadherin-6, and the glomerular epithelium that begins to express P-cadherin (Tassin et al., 1994). There is a small region of overlap between the cadherin-6 and E-cadherin

expression domains that is more prominent on the apical side of the epithelium. This overlap does not appear to be due to cell mixing, rather it appears that individual cells can express both E-cadherin and cadherin-6 simultaneously. Expression of cadherin-6 marks the differentiating and proliferating proximal tubule cells in the nephrogenic zone. In the newborn kidney, cadherin-6 expression is also high in the medullary zone as the ascending and descending limbs of Henle's loop grow towards the renal papilla. These ascending and descending limbs are probably the last epithelial structures formed in the developing nephron.

The *in vitro* antibody inhibition experiments clearly point to a function for cadherin-6 in the aggregation and/or polarization of induced mesenchyme. Induced Pax-2-positive metanephric mesenchyme appears loosely organized with little evidence of tubule formation when cadherin-6 blocking antibodies are added to kidney organ cultures. Although cadherin-6 is not expressed in the WT1-positive cells of the glomeruli, glomerular development is inhibited with the blocking antibodies most probably because the requisite steps leading to s-shaped body formation cannot occur. Cadherin-6 expression appears down-regulated in the presence of blocking Fab fragments and suggests that expression levels may be potentiated by aggregation. Alternatively, the Fab fragments that cover the extracellular domain of cadherin-6 may react poorly with the secondary antibody made against the whole rabbit IgG molecule.

Several lines of evidence support our interpretation that the cadherin-6 Fab fragments function by inhibition of cadherin-mediated cell-cell adhesion. First, the antigen used spans amino acids 68-410 of the cadherin-6 propeptide and includes most of the extracellular domain. The proposed N-terminal signal sequence and precursor peptide spans amino acids 1-53 (Xiang et al., 1994); the first CAD domain encompasses amino acid residues 54-159 and is followed by four additional CAD repeats. This amino terminal CAD domain is essential for homophilic interactions of class I cadherins (Nose et al., 1990). Although it is not clear whether the type II cadherins interact with similar affinities and sequence requirements, conserved elements within this amino terminal CAD domain can be identified. Our antigen includes 87% of the first CAD domain, including the conserved β D, β E and calcium-binding domain

(Overduin et al., 1996), the entire 2nd and 3rd CAD repeat and approximately half of the 4th CAD repeat. More importantly, the cadherin-6 antibodies are able to inhibit calcium-dependent aggregation of cadherin-6-expressing L cells. Mouse L cells do not express any endogenous cadherins, show little cell-cell contact in monolayers, and have been used extensively to assay cadherin function (Urushihara et al., 1979). Initial attempts to generate cadherin-6 transformants using plasmid expression vectors were unsuccessful, perhaps due to high levels of expression. Using retroviruses that integrate only a single copy of the mouse *cadherin-6* gene, we were able to generate several expressing lines and demonstrate cadherin-6-induced aggregation similar to what has been reported for chicken cadherin-6B (Nakagawa and Takeichi, 1995). Similarly, in cultured kidney rudiments exposed to cad6-Fab, induced metanephric mesenchymal cells are more spread out and found along the entire ureteric bud epithelium, whereas control mesenchymal cells are generally tightly associated and never found directly adjacent to mature ureteric bud epithelium.

The activation of cadherin-6 in the mesenchyme-derived renal vesicle precedes the establishment of epithelial polarity. Strikingly, expression of E-cadherin is sufficient in culture to redistribute the localization of cell surface proteins and reorganize the membrane cytoskeleton (McNeil et al., 1990). Whether cadherin-6 is a similar determinant of polarization in the renal vesicle epithelium or merely one protein among many that are necessary to form adhesive junctions remains to be determined.

In summary, the ability of cells to undergo cadherin switching may be potent driving force for morphogenesis. The data presented in this report demonstrate that cadherins can demarcate the newly formed mesenchyme-derived epithelial cells from the ureteric bud epithelium and surrounding interstitial mesenchyme. Furthermore, as this new epithelium proliferates into the s-shaped body, it becomes compartmentalized into glomerular, proximal tubular and distal tubular epithelium. Differential expression of cadherin-6, E-cadherin and P-cadherin may provide repellent forces that aid in cleft formation as well as pattern the developing nephron with respect to cell types along the proximal-distal axis.

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Note added in proof

A recent report by Rosenberg et al. (*Dev. Biol.* **187**, p. 55, 1997) demonstrates that the expression of R-cadherin is very similar to cadherin-6 in the developing kidney. R-cadherin is a type-I cadherin and does not react with our cadherin-6 antibodies. Thus, a role for R-cadherin in mesenchymal cell aggregation must also be considered.



Kidney Development in Cadherin-6 Mutants: Delayed Mesenchyme-to-Epithelial Conversion and Loss of Nephrons

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During nephrogenesis, dynamic changes in the expression of cell adhesion molecules are evident as epithelial structures differentiate from the induced mesenchyme. The cadherins are thought to play an important role in the metanephric mesenchyme, when cells aggregate to form the renal vesicle, a polarized epithelial structure which eventually fuses with the ureteric bud to generate a continuous nascent nephron. We have generated and analyzed mice with a targeted mutation in the gene encoding cadherin-6 (Cad-6), a type II cadherin expressed during early stages of nephrogenesis. These mice are viable and fertile, and they complete both early and late aspects of nephrogenesis. However, upon closer examination *in vitro* and *in vivo*, a fraction of the induced metanephric mesenchyme in Cad-6 mutant kidneys fails to form a fully polarized epithelium on schedule. Moreover, a significant number of the renal vesicles in Cad-6 mutant kidneys apparently fail to fuse to the ureteric bud. These alterations in epithelialization and fusion apparently lead to a loss of nephrons in the adult. These studies support the idea that cadherins play an essential role in the formation of epithelial structures and underscore the importance of timing in orchestrating the morphogenesis of complex epithelial tissues. © 2000 Academic Press

Key Words: cadherin-6; kidney development; epithelial polarization; epithelium fusion; renal tubules.

INTRODUCTION

The development of the embryonic kidney requires a series of reciprocal inductive interactions that lead to the assembly of nephric tubules (reviewed in Saxen, 1987; Vainio and Muller, 1997). These inductive events occur when a specialized region of intermediate mesoderm called the metanephric mesenchyme contacts an outgrowth of the Wolffian duct, the ureteric bud (Saxen, 1987). Mesenchymal cells respond to ureteric bud-derived inductive signals by aggregating and forming an epithelial structure called the renal vesicle, which then fuses with the ureteric bud to form a nascent nephron (Ekblom, 1989; Saxen, 1987). The

developing nephron then elongates by cell proliferation, and each region differentiates further into the various tubules that comprise a functioning nephron unit (reviewed in Lechner and Dressler, 1997). Among the hallmarks of the *de novo* assembly of epithelia from mesenchymal cells are aggregation and polarization, which may depend on the cadherin family of cell adhesion molecules (Takeichi, 1991; Schmidt *et al.*, 1993; Adams and Nelson, 1998; Cho *et al.*, 1998).

The cadherin superfamily consists of transmembrane proteins that mediate calcium-dependent cell adhesion and whose extracellular domains contain multiple copies of the so-called cadherin repeat (reviewed in Gumbiner, 1996; Takeichi, 1991). Cadherins can be subdivided into subfamilies based on structural features of the extracellular domain and functional properties of the intracellular domain. The classical or type I cadherins, which include E-, N-, P-, and R-cadherin, comprise a subfamily whose extracellular domains contain five cadherin repeats, the first of which is necessary for specific homophilic interactions. These se-

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quences promote interactions between cadherins of the same type on opposing cells (*in trans*) and possibly in the same cell (*in cis*) (Blaschuk *et al.*, 1990; Nose *et al.*, 1990; Patel and Gumbiner, 1995; Shapiro *et al.*, 1995). In addition, the type I cadherins encompass separate intracellular domains that mediate binding to the actin-based cytoskeleton via a group of linker proteins, the catenins (Kemler and Ozawa, 1989; Ozawa *et al.*, 1989, 1990; Hirano *et al.*, 1992), and *cis* interactions for clustering cadherins on the cell surface (Yap *et al.*, 1998; Katz *et al.*, 1998). Co-operativity achieved by interactions of cadherins on opposing cells *in trans*, along with the interactions in *cis*, is thought to generate a strong adhesive force that can drive selective cell-cell aggregation (Gumbiner *et al.*, 1988). In addition, the binding and clustering of cadherins into adhesive plaques appear to be the underlying basis of the adherens junction, a specialized site of cell-cell contact that binds cells in solid tissue (reviewed in Gumbiner, 1996). In epithelia, disruption of this junction has a profound effect on the maintenance of other junctional contacts and ultimately leads to a loss of epithelial polarity and integrity (Gumbiner *et al.*, 1988). Many of these features also apply to the type II subfamily of cadherins. Although less well characterized, Cad-6,⁴ cadherin-11, and F-cadherin are closely related to the classical cadherins in both structure and function (Cho *et al.*, 1998; Hoffmann and Balling, 1995; Kimura *et al.*, 1995; Espeseth *et al.*, 1995). The type II cadherins also mediate cell adhesion via a homophilic binding interaction and bind to the actin-based cytoskeleton via the catenins (Nakagawa and Takeichi, 1995; Inoue *et al.*, 1997). However, the type I cadherins are widely expressed in epithelial or neural tissue, whereas the type II cadherins are more cell type specific, particularly during embryonic development (Suzuki *et al.*, 1991, 1996; Takeichi, 1991). Nonetheless, the type I and type II cadherins share a number of properties that make them well suited for mediating the selective cell-cell interactions underlying tissue morphogenesis.

In the developing kidney, the uninduced metanephric mesenchyme expresses cadherin-11 while the ureteric bud expresses E-cadherin (Cho *et al.*, 1998). When the metanephric mesenchyme aggregates in response to inducing signals from the ureteric bud, the expression of cadherin-11 is down-regulated and the expression of R-cadherin and Cad-6 is up-regulated (Rosenberg *et al.*, 1997; Cho *et al.*, 1998). The onset of Cad-6 expression, in particular, is correlated with formation of the polarized epithelium and the renal vesicle and the deposition of a basement membrane. The renal vesicle differentiates further by forming the comma-shaped and then the S-shaped body. At the S-shaped body stage, R-cadherin is primarily restricted to the epithelial structures that are oriented farthest from the ureteric bud, E-cadherin is expressed in the bud and most distal tubules, while Cad-6 is found between the E-cad and

the R-cad domains in cells destined to become proximal tubules (Rosenberg *et al.*, 1997; Cho *et al.*, 1998). As tubulogenesis proceeds, Cad-6 becomes restricted to the developing proximal tubules, including the ascending and descending limbs of Henle's loop, and R-cadherin becomes restricted to the developing cells of the future Bowman's capsule and the adjoining proximal tubule cells (Rosenberg *et al.*, 1997; Cho *et al.*, 1998). As Cad-6 expression is down-regulated, E-cadherin becomes more prominent in the mature proximal tubules and P-cadherin is evident in the glomerular cleft and subsequent glomerular epithelium (Tassin *et al.*, 1994; Cho *et al.*, 1998). Thus, during kidney development, dynamic changes in the expression of the type II and type I cadherins correlate with tissue remodeling, suggesting strongly that cadherins mediate the differential cell adhesion required for proper nephrogenesis.

We have previously shown that inhibitory antibodies to Cad-6 will block the formation of comma- and S-shaped bodies when added to kidney rudiments cultured as explants, suggesting that Cad-6 is required in early nephrogenesis for the formation of epithelial structures (Cho *et al.*, 1998). To extend this analysis, the role of Cad-6 in the developing kidney was examined in mice lacking the Cad-6 protein, as generated by targeted gene disruption. Mice lacking Cad-6 are viable and fertile, and the histology of their kidneys is for the most part normal. However, in developing kidneys lacking Cad-6, a significant fraction of mesenchymal aggregates is delayed in their conversion into epithelial structures both *in vivo* and *in vitro*. Furthermore, mesenchymally derived tubules normally fuse to the ureteric bud to form a continuous epithelium but this event is delayed or fails to occur in a significant fraction of the developing nephrons in the absence of Cad-6. The delay in epithelialization and fusion apparently leads to a subset of nonfunctional nephrons as evidenced by necrosis of nephrons after birth and a significant reduction in the number of nephrons in the adult. These studies support the idea that cadherins play an essential role in the formation of epithelial structures during nephrogenesis and demonstrate that the precise coordination of morphogenetic events is critical for orchestrating the development of complex epithelial tissues.

MATERIALS AND METHODS

Generation of *Cad-6* Null Mice

Genomic sequences for Cad-6 were isolated from an isogenic Lambda FIX II SVJ/129 mouse genomic library (Stratagene) screened with the first 651 bp of *Cad-6* cDNA previously described (Cho *et al.*, 1998). One phage clone was isolated that contained the exon encoding the signal sequence along with the "pre" region and the first 27 amino acids of the first extracellular repeat. This clone was used to create the *Cad-6* targeting vector. The targeting construct was designed to insert PGK-*neo* into the first exon of the *Cad-6* gene, at amino acid 68, in the orientation opposite to that of the gene. The insertion of PGK-*neo* is downstream of the signal sequence necessary for targeting of mature Cad-6 into the mem-

⁴ Abbreviations used: Cad-6, cadherin-6; LTA, lotus tetragonolobus.

brane such that any spurious transcripts produced would not be targeted to the membrane. Additionally, the construct contains a PGK-*tk* cassette at the 3' arm of the construct to screen against nonhomologous recombination. ES cells (CCE; kind gift from L. Robertson) were maintained on neomycin-resistant/gancyclovir-resistant LIF-secreting STO fibroblasts (SNL fibroblasts; kindly provided by S. O'Gorman). ES cells were electroporated with 25 μ g of linearized vector DNA using standard settings (Bio-Rad Gene Pulser). After cells were cultured for 8–10 days in medium containing G418 (200 μ g/ml) and FLAU (0.2 μ M), 96 drug-resistant clones were picked and expanded for Southern blot analysis. ES cell clones were screened for correctly targeted homologous recombination using a 2.6-kb external genomic DNA probe on ES cell DNA cut with *EcoRI* (Fig. 1A). One correctly targeted clone (C34) was expanded, microinjected into C57/B6 host blastocysts, and reimplanted into ICR pseudo-pregnant females by standard procedures (Salk Institute Transgenic Mouse Facility). Chimeric offspring were bred to C57/B6 females and allelic transmission was gauged by coat color and subsequent Southern analysis. The Cad-6 null allele was maintained on a mixed 129SVJ/C57B6 background.

Genotyping

Genomic DNA was prepared from extraembryonic membranes or tail biopsies and used for Southern blotting or PCR. An internal primer for the Cad-6 gene (primer 1, 5' AAGCCTAGACTGTC-CAGACAC 3') and a primer specific for the neo gene (primer 3, 5' CCAGCCTCTGAGCCAGAAAG 3') were used to identify the mutated allele. Primer 1 and a primer specific for the Cad-6 gene 3' of the neo insertion site (primer 2, 5' CTCTTTTGGCTCGGC-CAGCCC 3') were used to identify the wild-type allele. The amplification conditions consisted of 35 cycles of 95°C for 1 min, 52°C for 1 min, 72°C for 1 min after starting with a denaturation step of 95°C for 5 min and ending with an extension step of 72°C for 5 min. Amplification across the neo gene by primers 1 and 2 was not seen in either heterozygous or homozygous animals presumably due to length limitations of the PCR under these conditions.

Generation of Cad-6 Antibodies and Western Analysis

To produce a rabbit polyclonal antibody to Cad-6, we immunized rabbits with an insoluble Cad-6 peptide (amino acids 128–598) made with an inducible bacterial expression vector (pQE system; Qiagen). This vector allows the Cad-6 peptide to be fused to an affinity tag made up of six consecutive histidine residues. After induction of protein expression, we purified the recombinant protein under denaturing conditions on a nickel column (Qiagen) according to the manufacturer. After stepwise dialysis in PBS, the protein was coupled to an activated resin column (Affi-Gel; Boehringer Mannheim) as per the manufacturer. This column was then used to purify crude serum collected from immunized rabbits. The specificity of this purified antibody was tested by immunohistochemistry on embryonic sections of WT and Cad-6 null mutants. The protein expression pattern was similar to the mRNA pattern deduced from *in situ* hybridization analysis; however, the sensitivity of this reagent was such that additional staining in axons and in spinal cord neurons was found (data not shown). The purified serum is specific for Cad-6 as immunohistochemistry and Western blot analysis of Cad-6 null kidneys reveal no immunopositive staining (data not shown and Fig. 1D). For Western analysis, protein extracts were prepared from dissected E18.5 kidneys that were

Dounce homogenized in SDS sample buffer and boiled. Proteins were separated by SDS-PAGE and blotted to Hybond-ECL membrane (Amersham). Blots were incubated with the Cad-6 antibody (1:500) and visualized by enzyme-linked chemiluminescence (Amersham).

Kidney Organ Culture

Kidney rudiments from E11.5 littermates were dissected, cultured, and stained as previously described (Cho et al., 1998). DNA was extracted from the anterior half of the embryo and genotyped by PCR as described above. After 48 h in culture, the number of ureteric bud tips was counted, as an indication of degree of development *in vitro*. Laminin-positive comma- and S-shaped structures were counted and the ratio of laminin-positive structures to bud tips was calculated.

Immunostaining

The kidneys of near-term and newborn animals were dissected out and the tail was used for genotyping. Tissues were immediately frozen in OCT (Sakura Finetechnical) in a dry ice/isopentane bath. Samples were cut on a cryostat and 20- μ m sections collected onto slides (PermaFrost Plus; Fisher). After air drying, sections were treated and stained as previously described (Cho et al., 1998). Whole-mount antibody staining of kidney organ cultures was as previously described (Cho et al., 1998). Staining with the anti Cad-6 antibody (1:250) was performed followed by a biotinylated goat anti-rabbit Ig (1:1000; Jackson Laboratories). Staining was visualized by addition of Cy3-conjugated streptavidin (1:1000; Jackson Laboratories). R-cadherin was a kind gift from M. Takeichi and was used as described (Rosenberg et al., 1997). Other antibodies and reagents were used at the following dilutions: Biotinylated goat anti-rat (1:1000; Jackson Laboratories); streptavidin-conjugated CY5 (Molecular Probes), 1:1000; laminin (Sigma; L9393), 1:200; ZO-1 (DSHB, University of Iowa), 1:250; LTA (Sigma), 1:100; and rhodamine phalloidin (Molecular Bioprobes), 1:500. For cases in which two antibodies derived from the same source animal were used, sections were first incubated with one antibody and then incubated with a FITC- or CY3-conjugated secondary antibody. After washing, the second primary antibody was applied and visualized with a biotinylated secondary antibody followed by streptavidin-conjugated CY5. Control stainings show that there is no spurious binding of the biotinylated secondary antibody to the first primary antibody applied (data not shown). Immunostained sections were visualized on a laser scanning microscope (Zeiss LSM 510) and colors assigned where appropriate.

Histological Preparation and Quantitation

Near-term embryos and newborn animals were isolated and their kidneys dissected out and fixed overnight in either 4% paraformaldehyde or 4% paraformaldehyde/0.1% glutaraldehyde. Kidneys were then prepared and embedded in JB-4 resin (Polysciences) or in Epon (Fluka). After embedding, 1- μ m serial sections were cut on an Ultracut E ultramicrotome (Reichert-Jung) and stained with toluidine blue or hematoxylin/eosin. After staining, the nephrogenic zone was examined for ureteric buds and their associated comma/S-shaped bodies. Samples were selected only if the lumina of the ureteric buds and comma/S-shaped bodies were visible. Suitable samples were then examined in each serial section for fusion. A positive fusion event was scored only when the

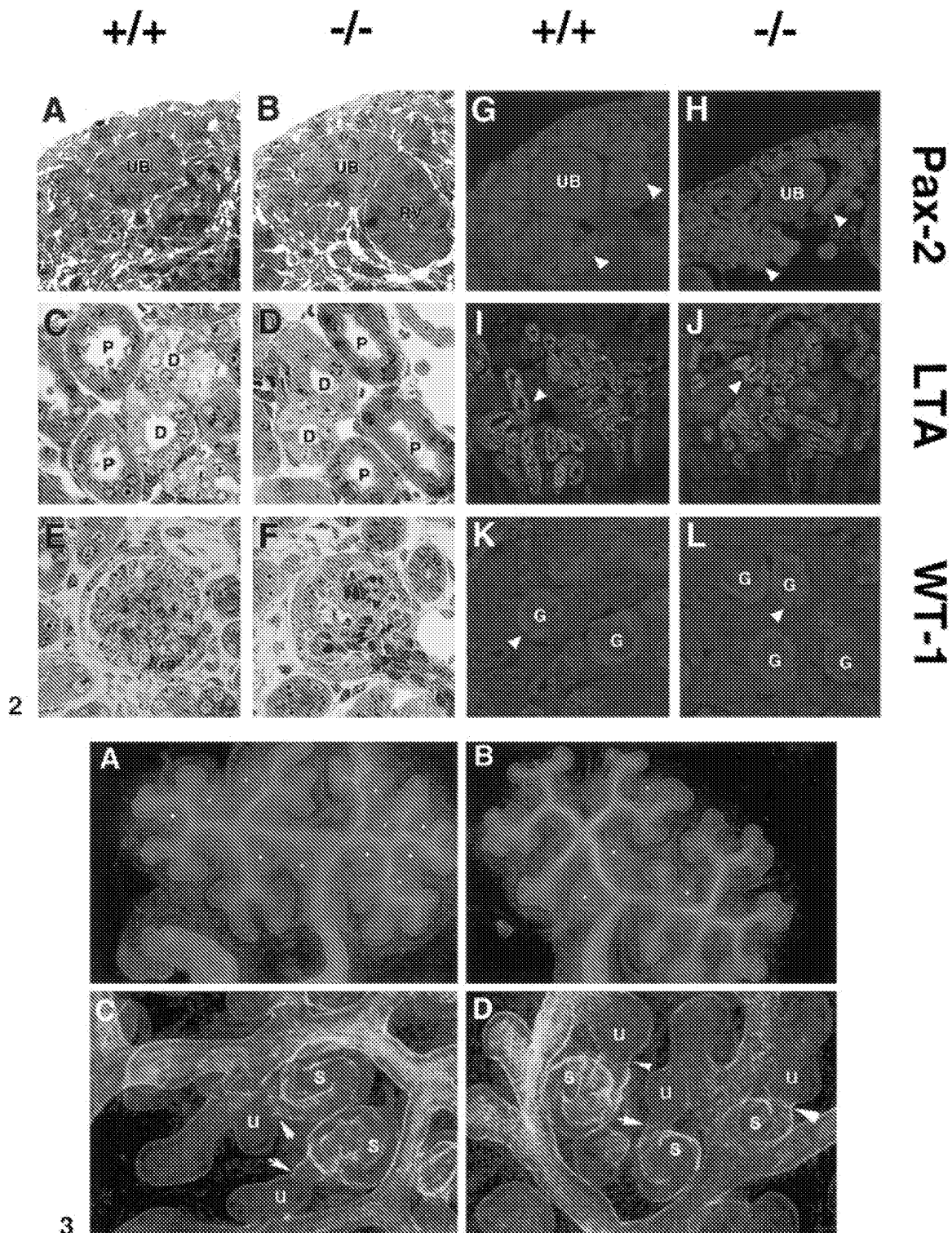


FIG. 2. Histological and molecular characterization of developing *Cad-6* null kidneys. (A-F) Plastic sections (1 μ m) from E18.5 kidneys stained with toluidine blue (A, B) Representative images taken in the nephrogenic zones of wild-type (A) and mutant (B) kidneys. Dashed lines outline the mesenchymal aggregates which are induced by the ureteric bud (UB) and then differentiate into the renal vesicle (RV). (C, D) Representative images taken of fully differentiated proximal (P) and distal (D) tubules from wild-type (C) and mutant (D) kidneys. (E, F)

mg/ml into the intraperitoneal space. Mice were sacrificed after 2 h. The kidneys from E16 embryos were removed, immersed in OCT, and flash frozen in liquid nitrogen. Sections were cut at 8 μ m and permeabilized with 2 N HCl, 0.5% Triton X-100, PBS for 1 min. Anti-BrdU (Sigma) was diluted 1:100 in PBS, 0.5% Tween 20, 2% goat serum. After incubation with TRITC-conjugated anti-mouse antibodies (Sigma) the sections were counterstained with 1 μ g/ml DAPI. Sections were rinsed three times and mounted with Gelvatol. Micrographs were taken with a Nikon ES800 using broad band filters. The total number of nuclei and BrdU-positive cells was counted over a given area. The proliferation index was calculated as the percentage BrdU-positive nuclei relative to the total number of nuclei in a section.

RESULTS

Generation and Characterization of *Cad-6* Null Mice

For homologous recombination, the *Cad-6* targeting vector was constructed by inserting a neomycin cassette into the exon encoding the amino terminus of *Cad-6* (Fig. 1A). After electroporation of the *Cad-6* targeting vector into CCE ES cells (kind gift from E. Robertson), selection in G418, and counterselection with FIAU, the 96 surviving cell colonies were screened by Southern blotting. The cell line C34 exhibited the predicted restriction digestion pattern indicative of correct homologous recombination. C34 was injected into C57/B6 host blastocysts, resulting in several male chimeras, one of which transmitted the mutation in the germ line. The founder mice (*Cad-6* $\pm$) were screened by Southern blot and PCR (Figs. 1B and 1C) to verify their genotype and subsequently used to generate a breeding colony. Mice that are homozygous for the targeted *Cad-6* allele did not express detectable amounts of *Cad-6* protein when tested by immunohistochemistry (data not shown) or by Western blotting (Fig. 1D), using a polyclonal rabbit antibody raised against the entire extracellular domain of *Cad-6* (Cho *et al.*, 1998). Although the targeted allele of *Cad-6* appears to result in a complete loss of *Cad-6* protein, both male and female *Cad-6* heterozygous and

homozygous animals are viable and fertile. Animals obtained from heterozygous crosses and heterozygous/homozygous crosses gave approximate Mendelian ratios. Heterozygous crosses produced viable offspring with genotypes that were 30% *Cad-6* $+/+$ (23/77), 49% *Cad-6* $\pm$ (38/77), and 21% *Cad-6* null (16/77), while crosses between heterozygous and homozygous mice produced viable offspring that were composed of 41.5% *Cad-6* $\pm$ (12/29) and 58.5% *Cad-6* null (17/29).

Kidney Development in *Cad-6* Mutants Is Grossly Normal

Mice lacking *Cad-6* were viable and fertile, suggesting that kidney function was not severely compromised. To determine the extent to which kidney development occurred normally in *Cad-6* mutant embryos, their kidneys were examined using standard histological methods, electron microscopy, and a variety of molecular probes. Superficially, almost all aspects of early nephrogenesis were evidently normal, including the aggregation and differentiation of the metanephric mesenchyme into comma- and S-shaped bodies (Figs. 2A and 2B) and the production of distal tubules, proximal tubules, loops of Henle, and glomeruli. Mutant and wild-type embryonic kidneys were indistinguishable by histology (Figs. 2C, 2D, 2E, and 2F and data not shown). Although *Cad-6* is expressed at high levels in developing proximal tubules (Paul *et al.*, 1997; Cho *et al.*, 1998), the polarized brush borders in the proximal tubules of *Cad-6* null kidneys were indistinguishable from those in control littermates (Figs. 2C and 2D). Additionally, electron micrographs of tight junctions and adherens junctions in the proximal tubules revealed no difference between mutant and control animals (data not shown).

The kidneys in *Cad-6* null animals were also examined using various molecular markers of kidney differentiation. Late embryonic kidneys (E18.5) were chosen, as they encompass a developmental profile from induced mesenchyme to fully differentiated tubules. Pax-2 is expressed by induced mesenchymal cells and is essential for epithelial

Representative images taken of mature glomeruli from wild-type (E) and mutant (F) kidneys. (G–L) Tissue sections from wild-type and *Cad-6* mutant kidneys were analyzed by immunofluorescence for the expression of tissue differentiation markers. (G, H) Pax-2 expression in mesenchymal aggregates (arrowhead) adjacent to UB in wild-type ($+/+$) and mutant ($-/-$) kidneys. (I, J) Lotus tetragonobolus (LTA) expression labels proximal tubules (arrowhead) where *Cad-6* expression also occurs normally. (K, L) WT-1 expression in podocytes (arrowhead) in the glomeruli (G) of wild-type (K) and mutant (L) kidneys. Expression patterns in both wild-type and mutant were in accordance with published expression studies of normal kidney tubular differentiation (see text).

FIG. 3. *Cad-6* null kidneys show reduced numbers of epithelial structures *in vitro*. *In vitro* explant cultures of wild-type and mutant kidney rudiments after 48 h of culture. Whole-mount immunofluorescent staining was performed on these explants using antibodies to laminin (red) and cytokeratin (green). Laminin marks the basement membranes of epithelializing structures, and cytokeratin labels the ureteric bud. Note the increased numbers of laminin-positive structures (dots) relative to the number of branched ureteric buds between wild-type (A) and mutant (B) explants. (C) High magnification of S-shaped structures (s) in wild-type kidneys stained for laminin. Note clean fusion (arrows) of ureteric bud epithelium (u) and S-shaped body (s). (D) High magnification of *Cad-6* $-/-$ kidney culture. Note the continuous laminin-positive fibers (arrows) between S-shaped body and ureteric bud epithelium in some developing tubules (arrowhead), whereas another fused region appears more like wild-type (arrows).

conversion (Rothenpieler and Dressler, 1993; Torres et al., 1995). The pattern of Pax-2 expression in the induced mesenchymal cells of Cad-6 null embryos was indistinguishable from that in wild-type controls (Figs. 2G and 2H), suggesting that Cad-6 is not necessary for the early reciprocal inductive interaction between the ureteric bud and the mesenchyme. Cad-6 is expressed mainly in cells of the developing kidney that are destined to become proximal tubules (Paul et al., 1997; Cho et al., 1998) and that are marked by the expression of lectin binding sites for lotus tetragonolobus (Laitinen et al., 1987). The pattern of LTA expression in the developing kidneys of Cad-6 null mice did not noticeably differ from that in control littermates (Figs. 2I and 2J), indicating again that Cad-6 is not necessary for the establishment or maintenance of the proximal tubules. Cad-6 is also dynamically regulated in the developing glomerulus. To assay for defects in the glomeruli, we examined the expression of the transcription factor WT-1, a marker of podocyte precursor cells in Cad-6 null kidneys (Ryan et al., 1995). The expression of WT-1 was unaffected (Figs. 2K and 2L) and histologically, the embryonic glomeruli were normal (Figs. 2E and 2F). Thus, loss of Cad-6 function in the developing kidney did not result in obvious cellular defects, in either early or late aspects of tubule morphogenesis.

In Vitro Development of Cad-6 Mutant Kidneys

In previous experiments, we used cultured kidney rudiments isolated from E11 embryos to assay kidney morphogenesis and showed a marked reduction in the formation of mesenchymal aggregates *in vitro*, when these rudiments were treated with Cad-6 inhibitory antibodies (Cho et al., 1998). Since these results were at apparent odds with those obtained with the Cad-6 mutant mice, we next examined the growth and differentiation of Cad-6 mutant kidneys in organ culture. Wild-type and mutant kidneys were isolated from E11 embryos, placed in culture for 48 h, and scored for several aspects of nephrogenesis using whole-mount immunohistochemistry. Anti-pancytokeratin staining marks the status of the ureteric bud epithelium, which branches extensively after 48 h to generate 30–40 bud tips (Saxen, 1987; Cho et al., 1998). Anti-laminin staining marks the basal lamina of the polarized epithelium of the ureteric bud and the mesenchymal-derived structures which form at the tips of bifurcated ureteric buds (Ekblom et al., 1980, 1990; Ekblom, 1989). Based on these assays, control explants after 48 h of culture have undergone ureteric bud branching and formed mesenchymally derived epithelia (Fig. 3A). Similarly, kidney explants from Cad-6 null mice also undergo extensive ureteric bud branching after 48 h in culture (Fig. 3B), indicating that the reciprocal interactions between the ureteric bud and the mesenchyme do not require Cad-6 function. In contrast, the formation of mesenchymally derived epithelial structures in the explanted mutant kidneys was significantly compromised. Based on the number of laminin-positive structures, both comma- and S-shaped

TABLE 1

Extent of Mesenchyme-to-Epithelial Conversion in Kidney Organ Cultures

Genotype	n	No. of comma- and S-shaped bodies/No. of ureter bud tips
+/, +/–	21	0.37 ± 0.12
–/–	25	0.22 ± 0.13 (P<0.001)

bodies, the formation of polarized epithelia was clearly reduced in Cad-6 null kidneys (Table 1). Cad-6 null kidney explants contained approximately 40% fewer laminin-positive, mesenchymally derived structures compared to wild-type and heterozygous kidney explants. Furthermore, well-developed S-shaped structures found in Cad-6 null kidneys were often located farther from the tips of the ureteric buds and frequently had laminin-positive fibers separating the mesenchymal-derived structure from the bud epithelium (Figs. 3C and 3D), indicating that these tubules are not continuous. Thus, these data suggest that the lack of Cad-6 function *in vitro* compromises the conversion of mesenchymal aggregates into polarized epithelia and disrupts, in some cases, the fusion of mesenchymal-derived epithelium to the ureteric bud epithelium.

Altered Laminin Staining and Fusion in Cad-6 Mutant Kidneys in Vivo

Based on the observations in explant culture, we analyzed the Cad-6 mutant kidneys further to determine whether similar alterations occur *in vivo* when mesenchymal aggregates epithelialize and fuse to the ureteric bud. As described above for the explant assays and shown more extensively in Fig. 5, we used the status of laminin staining (discontinuous versus continuous) to score the conversion of metanephric mesenchyme into epithelial structures in the kidneys of both Cad-6 mutants and their wild-type littermates. In this analysis, laminin staining was scored only in mesenchymal aggregates induced by ureteric buds at the same developmental age (bifurcated ureteric bud stage) in order to compare events that occur in control and mutant kidneys at the same period of nephrogenesis. This stage was chosen because it is the approximate time when induced aggregates become a fully polarized epithelial renal vesicle (Lechner and Dressler, 1997). The results show that the number of aggregates with continuous laminin staining was markedly reduced in Cad-6 –/– kidneys. Only 25% of all aggregates in Cad-6 mutant kidneys had continuous laminin staining, compared to 53.5% of all aggregates in control kidneys (Fig. 4A). Thus, these data indicate that, *in vivo*, a subset of mesenchymal aggregates is delayed or fails to develop normally into epithelial structures in the absence of Cad-6.

We next asked whether the fusion of the S-shaped bodies to the ureteric buds was affected in Cad-6 mutant kidneys *in vivo* as observed *in vitro*. For this analysis, the nephro-

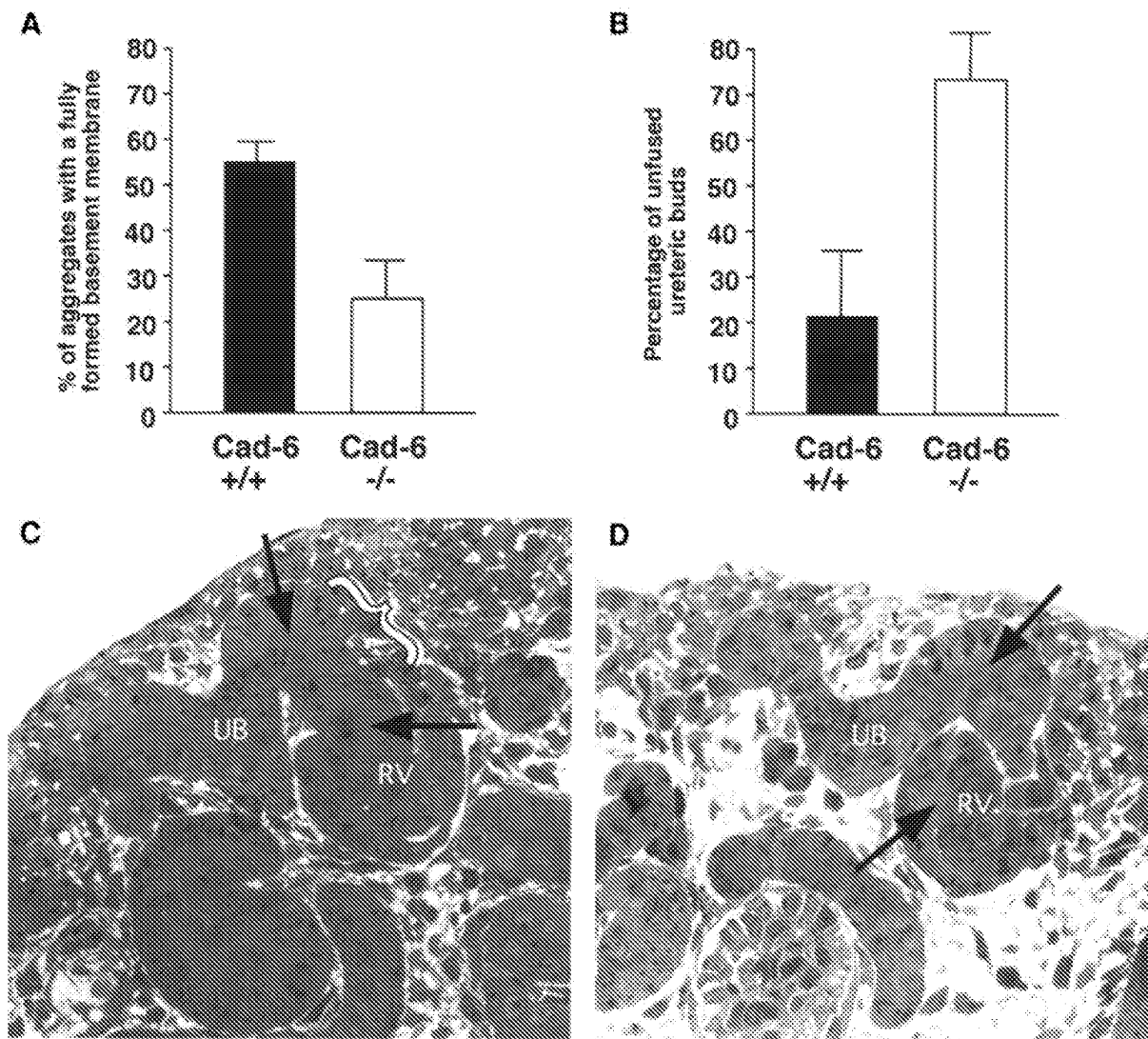


FIG. 4. Alterations in laminin staining and fusion in *Cad-6* null kidneys. (A) Frozen sections of developing kidneys from *Cad-6* null embryos (E14–15.5) and control littermates were stained with antibodies to laminin and scored only for the presence of a fully formed basement membrane. Laminin is initially deposited at the basal lamina in thin discontinuous strips which become thicker and form a continuous ring around the basal surface of the epithelial structure when a basal lamina forms (see Figs. 5D–5F). Data were normalized between control and mutant kidneys by sampling from aggregates induced by ureteric buds at the same developmental stage (bifurcated ureteric bud stage). Data are from five *Cad-6* null embryos and five control littermates (approximately 100 kidney sections each) and represent the means $\pm$ SEP ($P < 0.001$, χ^2 statistic with Yate's correction). (B and C) The nephrogenic regions of E18.5 kidneys from *Cad-6* null animals and control littermates were examined in toluidine blue-stained serial plastic sections (1 μ m) for fusion events. (B) Quantitation of fusion as defined by the status of the lumina and epithelial cell polarity in serial sections from mutant and control kidneys. Approximately 10 different regions were scored by serial section analysis in each kidney. Data represent the averages from five mutant animals and five control littermates and show the means $\pm$ SEP ($P < 0.001$, χ^2 statistic with Yate's correction). (C) Example of a positive fusion event in a control kidney. Note that the lumina of the ureteric bud (UB) and renal vesicle (RV) are contiguous and that the cells at the fusion site are ordered in a typical epithelial fashion (bracket), indicating that the two tubule subtypes have indeed fused to form a continuous tubule. (D) Example of an unfused tubule from a mutant littermate. Note that the lumina of both UB and RV are visible, indicating that this section is through the midpoint of each tubule subtype, but that the two structures are not fused.

genic zone of E18.5 kidneys was assayed by determining, in serial plastic sections, the percentage of S-shaped bodies not fused to ureteric bud. In wild-type kidneys, 21% of the

ureteric buds were scored as being unfused to mesenchymally derived epithelia by our parameters (Figs. 4B and 4C). In contrast, 73.3% of ureteric buds were not fused to

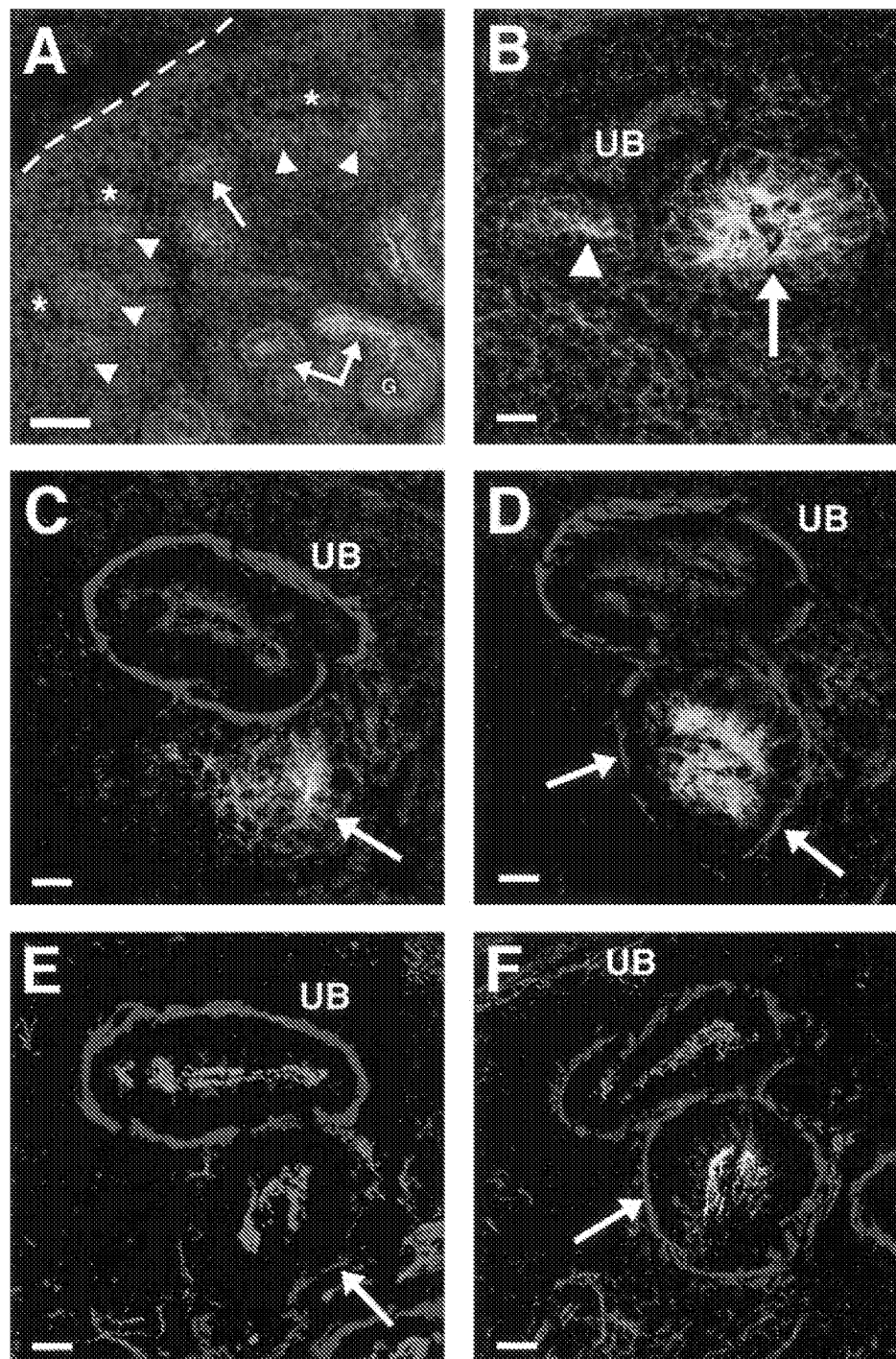


FIG. 5. Immunostaining of cadherins and junctional proteins during the mesenchymal-to-epithelial transition. Shown are confocal images of representative sections from the nephrogenic zone of developing kidneys from wild-type (A–D, F) and mutant (E) E14–15.5 embryos. (A) Immunofluorescence analysis of R-cadherin (green) and Cad-6 (red) in the nephrogenic zone. The dashed line represents the border of the developing kidney. Note that R-cadherin expression precedes Cad-6 expression in the initial mesenchymal aggregates (arrowheads) formed in response to ureteric bud (asterisk) signaling. Double-headed arrow indicates a developing proximal tubule which expresses only Cad-6 more distally (red signal) and a more proximal part of the tubule which expresses both Cad-6 and R-cadherin (yellow signal). Note that the developing glomeruli express only R-cadherin (green signal, G). The single arrow shows an R-cadherin-positive aggregate which is associated with a ureteric bud out of the section plane. (B) More mature aggregate stained as in (A) which expresses both Cad-6 and

comma- or S-shaped bodies in *Cad-6* null kidneys (Figs. 4B and 4D). Thus, fusion of the mesenchymal-derived epithelium to the ureteric bud also does not occur as efficiently *in vivo* in absence of *Cad-6*.

Developmental Analysis of Cadherin-6 Mutants

The results described above indicate that the loss of *Cad-6* may reduce the proportion of mesenchyme aggregates that form a polarized renal vesicle and subsequently fuse to the ureteric bud. To characterize this phenotype further, we determined more precisely when *Cad-6* is expressed during the formation of the renal vesicle relative to expression of proteins that mark aggregation and the formation of a polarized epithelium. First, we examined the expression of R-cadherin, which is also present in the pretubular mesenchymal aggregates in a pattern similar to that of *Cad-6* (Rosenberg *et al.*, 1997). Using double immunofluorescence labeling, *Cad-6* expression was never seen in early mesenchymal aggregates without R-cadherin coexpression (Figs. 5B and 5C), demonstrating that R-cadherin expression precedes that of *Cad-6* in mesenchymal aggregates that are induced by the ureteric bud (Fig. 5B). Since *Cad-6* expression occurs after that of R-cadherin, we next determined whether it occurs before or after the aggregates undergo polarization as measured by the formation of junctional complexes and the deposition of a well-organized basement membrane. To mark the formation of junctional complexes, we stained kidneys with antibodies directed against ZO-1, a constituent of zona occludens (ZO), while the formation of a basement membrane was marked by staining with laminin as described above. When kidneys were triple labeled with antibodies directed against R-cadherin, ZO-1, and laminin, mesenchymal aggregates were detected that stained with R-cadherin alone, with both R-cadherin and ZO-1, or with all three markers, but not with any other combination of markers (Figs. 5C, 5D, and 6A). This result confirms that R-cadherin precedes the expression of ZO-1, which in turn precedes that of laminin. When wild-type kidneys (E14–15.5) were triple labeled with antibodies directed against *Cad-6*, ZO-1, and laminin, mesenchymal aggregates were detected that double labeled with *Cad-6*/ZO-1 or triple labeled with *Cad-6*/ZO-1/laminin, but not with any

other combination of markers, suggesting that *Cad-6* and ZO-1 expressions coincide and precede that of laminin (Figs. 5F and 6A). In contrast, *Cad-6* mutants had a higher proportion of mesenchymal aggregates that were ZO-1 positive but had not formed a well-defined basement membrane, as judged by laminin staining (Fig. 5E).

The expression pattern of *Cad-6* indicates that it may function between the early aggregation of mesenchymal cells and the formation of a polarized epithelium. To test this possibility, we compared the proportion of mesenchymal aggregates that express ZO-1, R-cadherin, and laminin in wild-type versus *Cad-6* mutant kidneys. The results show that there is no statistical difference between wild-type and mutant kidneys in the relative percentages of aggregates which express solely R-cadherin (Fig. 6B). This finding supports previous data suggesting that there is no effect on initial mesenchymal aggregation (Figs. 4A and 4B). However, the proportion of aggregates that stained with ZO-1/R-cadherin or triple labeled with laminin/R-cadherin/ZO-1 was significantly different between developing mutant and wild-type kidneys (Fig. 6B). While the relative percentage of R-cadherin- and ZO-1-expressing aggregates was increased in *Cad-6* null kidneys, the percentage of aggregates expressing R-cadherin and ZO-1 and having a complete basement membrane was decreased (Fig. 6B). This finding strongly suggests that mesenchymal aggregates from *Cad-6* null kidneys are delayed in their ability to make the transition from epithelializing aggregate to a fully polarized epithelial structure. This finding is consistent with the onset of *Cad-6* expression in the mesenchymal aggregates which follows that of R-cadherin and coincides with the formation of junctional complexes marked by the expression of ZO-1. Interestingly, *Cad-6* expression precedes the deposition of a basement membrane as marked by laminin staining and the loss of *Cad-6* function delays the transition from a ZO-1⁺/laminin⁻ to a ZO-1⁺/laminin⁺ epithelium.

If the proliferation rate of the induced mesenchymal aggregates were reduced, it is possible that the critical mass required for formation of a fully polarized renal vesicle would not be reached, thus resulting in the phenotype observed. Also, the window of opportunity for comma-shaped bodies to fuse with the branching ureteric bud might

R-cadherin as seen by the yellow signal (arrow). Note that this aggregate is older than the aggregate in (A) and has begun to polarize and form a lumen. The ureteric bud (UB) is negative for both *Cad-6* and R-cadherin. The arrowhead indicates a glancing section through a similarly stained aggregate. (C) Triple immunofluorescence staining of R-cadherin (green), ZO-1 (red), and laminin (blue) in a preepithelial mesenchymal aggregate. The ureteric bud is ringed by laminin (blue) and is stained with ZO-1 (red), indicating that it is a fully epithelialized structure. Note that the aggregate is R-cadherin and ZO-1 positive (yellow staining, arrow), but has not yet laid down a basement membrane. (D) Epithelializing aggregate stained as in (C). Note that this aggregate is in the initial stages of basement membrane formation as indicated by the discontinuous and weak laminin staining around the periphery of the aggregate (arrows). (E) Triple immunofluorescence staining of *Cad-6* (red), ZO-1 (green), and laminin (blue) in an epithelializing aggregate of a mutant kidney. Note that this aggregate is *Cad-6* negative and ZO-1 positive and has begun to polarize and form a lumen. Note that the basement membrane as indicated by the laminin staining around the periphery of the aggregate (arrow) has not yet fully formed. (F) Fully epithelialized renal vesicle from a wild-type kidney, stained as in (E), which has formed a complete basement membrane as seen by the strong continuous laminin staining along the basal surface (arrow).

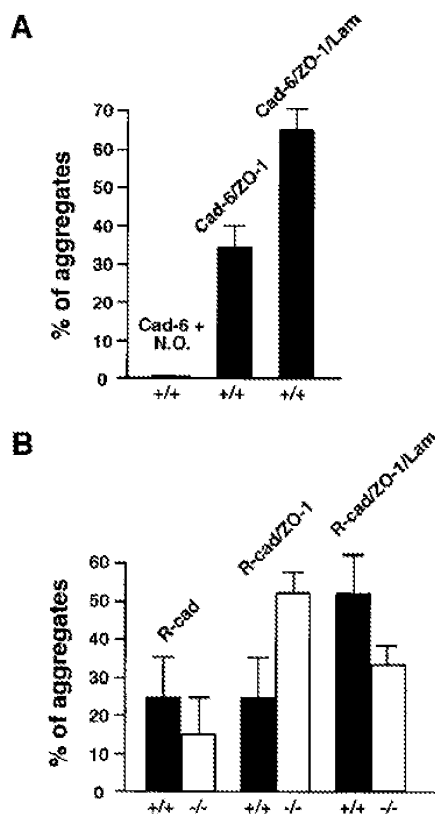


FIG. 6. Hierarchy of expression of cadherins and epithelial markers during the mesenchyme-to-epithelial transition *in vivo*. The expression patterns of Cad-6 and R-cadherin were determined relative to those of junctional markers (ZO-1 and laminin) as in Figs. 5C–5F. (A) Wild-type kidney (E14–15.5) mesenchymal aggregates were scored based upon their expression profiles of laminin, R-cadherin, and ZO-1 and the results displayed as a relative percentage of total aggregates. Data represent the averages of 8–12 kidney sections from five control animals $\pm$ SD. Aggregates were scored as laminin positive if they had strong continuous laminin staining indicating that a complete basement membrane had formed (Fig. 5F). N.O., not observed. (B) Cad-6 null kidneys and control littermates (E14–15.5) were stained with antibodies to R-cadherin, ZO-1, and laminin and scored as in (A). Note that there is no statistical difference in the relative percentages of R-cadherin-positive aggregates between control (solid bars) and mutant kidneys (open bars, $P > 0.2$, Student's *t* test). However, the relative percentage of R-cadherin- and ZO-1-expressing aggregates is increased in Cad-6 null kidneys ($P < 0.002$, Student's *t* test) and the relative percentage of aggregates expressing R-cadherin and ZO-1 and having a complete basement membrane is decreased in Cad-6 mutants ($P < 0.008$, Student's *t* test). This indicates that mesenchymal aggregates from Cad-6 null kidneys are delayed in forming fully polarized epithelial structures. Data represent the averages of 8–12 kidney sections from five mutant animals and five control littermates $\pm$ SD.

be missed if proliferation were affected. In order to examine this possibility, incorporation of BrdU was assayed in developing embryos at late stages. After a 2-h *in vivo* exposure

to BrdU, E16 kidneys were stained with anti-BrdU antibodies and the number of positive nuclei per unit area was assessed (Fig. 7). On average, an area containing 180 cells was counted. Percentages BrdU-positive cells were calculated as 40 ± 8 and $45 \pm 7\%$ for wild-type and Cad-6 mutants, respectively ($n = 10$). These numbers were not significantly different. Thus, the delay in formation of fully polarized epithelia observed in Cad-6 mutants is not due to reduced cell proliferation and supports the hypothesis that loss of function of Cad-6 results in a delay in the mesenchymal-to-epithelial transition of mesenchymal aggregates during nephrogenesis.

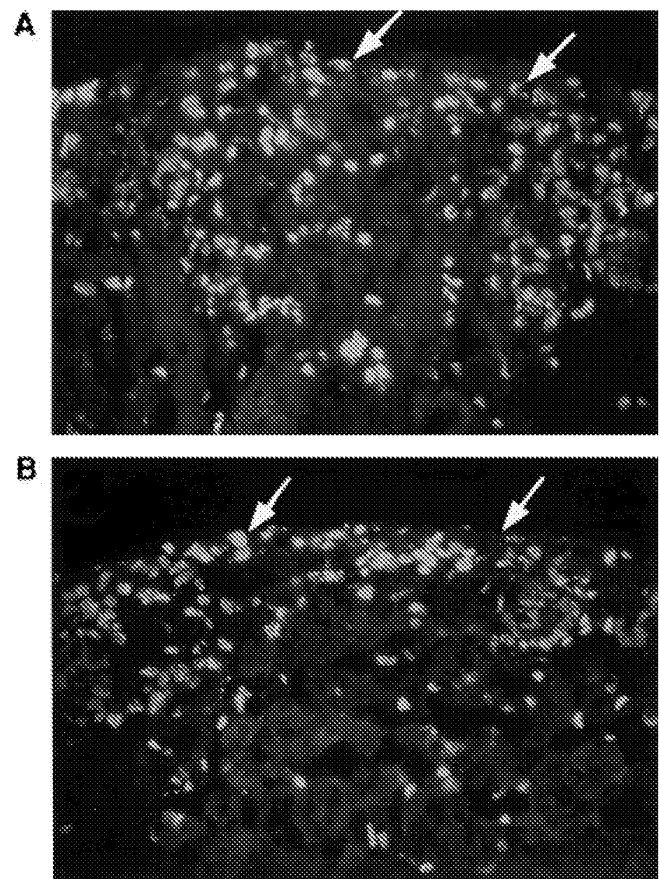


FIG. 7. Cellular proliferation in wild-type and Cad-6 mutant kidneys. After a 2-h pulse of BrdU, *in vivo* incorporation was examined by anti-BrdU antibodies in E16.5 kidneys. (A) Wild-type kidney showing BrdU incorporation in the nephrogenic zone along the periphery (arrows) as well as in the developing tubules in the cortical region. (B) Similar section through a Cad-6 mutant kidney showing indistinguishable levels of BrdU incorporation. BrdU-positive cells were counted in equivalent areas of 10 different sections. Sections were counterstained with DAPI and the total number of nuclei was determined. Percentages BrdU-positive cells were 40 ± 8 and $45 \pm 7\%$ for wild-type and Cad-6 mutants, respectively ($n = 10$).

Loss of Nephrons in *Cad-6* Mutant Kidneys

The analysis described above indicates that a subset of renal vesicles is delayed in their development as marked by laminin staining and may fail to fuse on schedule with the ureteric bud. To determine if these defects result in a reduction in functioning nephrons, we scored the number of nephrons in the kidneys from adult and embryonic wild-type and mutant animals. In adult mutant animals, there were approximately one-third fewer nephrons, based on the number of glomeruli, per unit area compared to wild type (Fig. 8). To determine if this loss of nephrons occurs pre- or postnatally, similar quantification studies were performed on newborn (P0) kidneys. There was no significant difference between the numbers of nephrons per unit area in P0 kidneys from *Cad-6* null animals and those of their wild-type littermates ($P < 0.5$, Student's *t* test; data not shown). However, in postnatal day 1 animals (P1), when filtration increases dramatically, a subset of nephrons from mutant kidneys became necrotic (Figs. 8B and 8D). In P1 wild-type kidneys, 7.4% of mature nephrons were scored as necrotic; in contrast, in homozygous null littermates, 22.7% of the mature nephrons were necrotic (Fig. 8D). The loss of nephrons did not appear to be due to apoptosis since TUNEL staining was not associated with the necrotic tissue (data not shown). Given the number of nephrons that are lost in mutant adult kidneys, we may be seeing only a fraction of the nephrons at P1 which will necrose or have already necrosed. Additionally, a number of necrotic nephrons also exhibited fluid-filled Bowman's spaces and dilated proximal tubules, suggesting that necrosis was occurring shortly after the onset of filtration and may be due to blocked tubules that failed to fuse correctly in the embryonic kidney.

DISCUSSION

Null phenotypes have been generated for the type I cadherins, E-cadherin and N-cadherin, by gene targeting, resulting in devastating consequences to early development in both mutants. E-cadherin mutant embryos arrest at the preimplantation stage, due to a loss of polarity and adhesion of cells in the early modulate (Larue *et al.*, 1994). While N-cadherin mutant embryos complete early stages of development and partially gastrulate, they show severe defects in the neural tube, heart, and other tissues where N-cadherin is expressed (Radice *et al.*, 1997). These severe defects observed in the N-cadherin and E-cadherin null mutants presumably reflect the widespread expression of these molecules in neural tissue and epithelium during normal development. In contrast, *Cad-6* has a more restricted expression pattern, in the kidney, in a subset of neural crest cells and their derivatives, and in the developing neural tube (S. P. Mah and C. Kintner, unpublished observations; Nakagawa and Takeichi, 1995; Inoue *et al.*, 1997). Although no detectable neural abnormalities were observed, subtle defects are prevalent in the developing kidneys of *Cad-6* null mice that result in a significant loss of nephrons. This reduction in nephron number is not sufficient to compro-

mise renal function, but does provide key insights into the role of the type II cadherins in tissue morphogenesis.

Several lines of evidence indicate that the loss of nephrons in the postnatal *Cad-6* mutant is due to blocked proximal tubules that arise when a fraction of renal vesicles fail to fuse on schedule with the ureteric bud. First, necrosis is observed only after birth at the time when there is a sharp increase in glomerular filtration. This necrosis is accompanied by dilated tubules and Bowman's spaces, indicating fluid buildup within the proximal part of the nephron (Figs. 8B, 8D, and 8E). Second, serial sections through embryonic kidneys clearly indicate a failure to form a continuous epithelial lumen in a significant fraction of developing nephrons (Fig. 4B). This fraction correlates well with the fraction of renal vesicles that are retarded in epithelialization as measured by the deposition of a complete basal lamina (Fig. 4A). These data suggest that the coordinated fusion of the renal vesicle and the ureteric bud to form a contiguous epithelium may be disrupted in the absence of *Cad-6*, thus resulting in blocked and nonfunctional nephrons. This model is consistent with the observation that the development of the glomeruli and proximal tubules appears normal in histological sections from late stage embryos. In *trans*-filter induction experiments, the mesenchymal-derived epithelium, once induced, will form differentiated tubules despite not being fused to the ureteric bud epithelium (Saxen, 1987). Thus, we would predict that if fusion does not occur, the histology of the kidney will be normal until glomerular filtration begins, at which time nonfused tubules will become dilated and necrotic. As *Cad-6* is expressed in the Bowman's capsule and proximal tubules (Paul *et al.*, 1997; Cho *et al.*, 1998), we cannot rule out the possibility that the loss of structural integrity and subsequent necrosis of these structures are directly due to loss of *Cad-6* at later stages. However, this is unlikely as tight junctions and adherens junctions form normally in mutant kidneys and are indistinguishable from wild-type junctions at the electron microscope level (data not shown).

Unfortunately we have not been able to demonstrate blocked tubules directly by retrograde labeling due to our inability to catheterize the small ureter in newborns. Similarly, efforts to characterize defects in kidney function via measurements of creatinine and serum protein levels are hampered by the fact that only a small subset of nephrons is affected in *Cad-6* mutant kidneys. Renal function must be seriously compromised (80–90% loss of function) to routinely detect differences in blood or urine samples.

Role of *Cad-6* in Vitro

The *in vivo* data presented in this report are in contrast to previous finding using the *in vitro* organ culture model and blocking antibodies to *Cad-6* (Cho *et al.*, 1998). Purified antibodies to the extracellular domain of *Cad-6* can inhibit calcium-dependent adhesion of *Cad-6*-transformed L cells and inhibit formation of mesenchymal aggregates and subsequent tubule formation in cultured E11 kidney rudi-

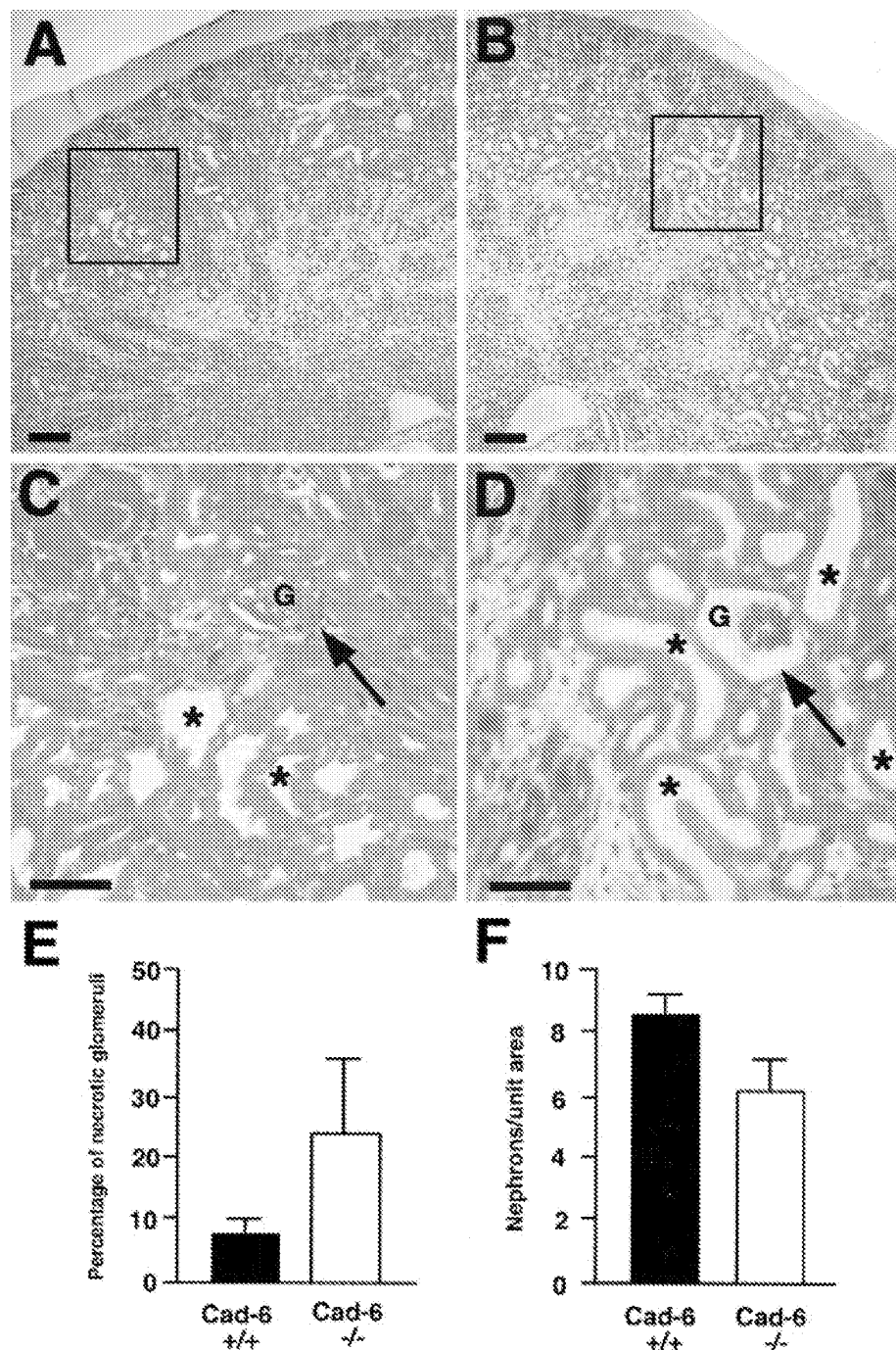


FIG. 8. Necrosis occurs in a subset of postnatal tubules. Postnatal day 1 pups (P1) and adult animals (6–18 months) lacking *Cad-6* and control animals were embedded in JB-4 resin and paraffin, respectively, sectioned (1 and 10 μ m, respectively), and stained with hematoxylin/eosin. Control P1 kidney (A, C) show typical glomeruli (G) and proximal and distal tubules. *Cad-6* mutant P1 kidneys (B, D), however, show evidence of glomerular “ghosts” (G), which indicate that necrosis of the glomerulus has occurred. Necrotic tubules are also in evidence (asterisks). Also note the increased prevalence of dilated tubules and Bowman spaces. (E) Mutant kidneys from P1 animals have 15% more necrotic glomeruli than control animals. Data represent the averages of 10–20 kidney sections from three mutant animals and three control littermates $\pm$ SD ($P < 0.001$, Student’s *t* test). As necrotic cells are removed very rapidly from the body, the low number of necrotic glomeruli/tubules we observe in mutant P1 kidneys may represent only a subtraction of the total number of glomeruli/tubules that will necrose or have already done so. (F) In adult animals, we see that there is a reduction of 34% of nephrons in *Cad-6* mutant kidneys

ments. Although somewhat delayed, aggregation and tubule differentiation appear relatively normal in *Cad-6* mutant mice *in vivo*. Cultured kidneys from *Cad-6* mutants also exhibit a delay in the formation of fully polarized, laminin-positive epithelial structures, but not nearly to the same degree as reported with the antibody blocking experiments. Thus, the effects observed with blocking antibodies are qualitatively similar but certainly more exacerbated when compared to the null phenotype *in vitro* and *in vivo*. Antibody inhibition studies are plagued by the possibility of steric hindrance whereby antibodies inhibit the function of molecules other than those of interest because of their close proximity or potential cross-reactivity. Furthermore, although the explant cultures can be used to assay a limited window of development, growth and differentiation are slower *in vitro* and may limit compensatory mechanisms from taking full effect (Saxen, 1987). In a recent characterization of the laminin $\alpha 5$ chain mutant phenotype, kidney organ culture of mutant animals also exhibited more severe defects, perhaps because of the suboptimal growth environment and the limited burst of proliferation observed *in vitro* (Miner and Li, 2000).

One possible compensating mechanism is R-cadherin, which is expressed in the mesenchymal aggregates *in vivo* prior to *Cad-6* and precedes the formation of other junctional complexes that are marked by the localized staining of ZO-1. We have examined the expression of E-cadherin and R-cadherin in *Cad-6* mutant kidneys at E13.5, E15.5, and E18.5 but have not detected any obvious change that could lead to compensation for the loss of *Cad-6* (data not shown). However, the overlapping expression of R-cadherin in early mesenchymal aggregates suggests that it may compensate partially for the loss of *Cad-6*. Also, the possibility of additional, as yet unidentified, type II cadherins expressed in overlapping domains cannot be ruled out. Taken together, results from both antibody inhibition experiments and *Cad-6* mutant organ cultures suggest that explant cultures are more susceptible to a loss of *Cad-6* function, perhaps because of a reduced rate of proliferation and differentiation or the more limited compensation of other adhesive systems that underlie the formation of the epithelialized renal vesicles.

Role of *Cad-6* in Vivo

During the mesenchyme-to-epithelial conversion, R-cadherin may promote the formation of zona adherens in the renal vesicle, leading to the establishment of junctional complexes. Moreover, *Cad-6* may affect the formation of

adherens junctions qualitatively or quantitatively, prior to the deposition of a basement membrane. In tissue culture cells, the formation of the zona adherens is a key regulatory step in the establishment of epithelial morphology. Gumbiner and colleagues (1988) have shown that disrupting cadherin function in MDCK monolayers by removing calcium from the culture medium results in a dispersal of ZO-1 and the rhodamine phalloidin-staining actin filaments. Reassembly of these junctions by readdition of calcium can be delayed with the addition of E-cadherin blocking antibodies. Similarly nonepithelial cell lines transfected with E-cadherin form adherens junctions and thereby acquire other properties of polarized epithelia (McNeill *et al.*, 1990). Thus, the results of *Cad-6* mutations on the epithelial-mesenchymal transition in developing kidneys are consistent with the hypothesis that the transition depends largely on the number and strength of contacts made via the cadherin-based adhesion system.

The Timing of Assembly of Epithelial Tubules

One of the striking features of nephrogenesis is the coordinate development of the ureteric bud and the mesenchymally derived epithelium through a series of reciprocal, inductive interactions (reviewed in Lechner and Dressler, 1997; Vainio and Muller, 1997). These interactions culminate with the fusion of ureteric bud-derived and mesenchymal-derived epithelium to form the continuous tubule of a nascent nephron. One would predict that a defect in one of these two interacting components during the early stages of nephrogenesis might prevent later aspects of nephrogenesis from occurring normally, particularly at the fusion stage. Consistent with this, the *Cad-6* null mice show a marked increase in the number of ureteric buds that are not fused to a mesenchymally derived tubule. Failure to complete the mesenchymal-to-epithelial transition on schedule in the absence of *Cad-6* significantly compromises the fusion of the mesenchymal-derived tubules with the ureteric bud. Thus, the branching bud continues its growth outward along the radial axis of the kidney before the connection can be established. Indeed, we observe that in *Cad-6* null kidneys, unassociated ureteric buds appear to grow back toward the medullary region of the kidney (data not shown).

Alternatively, *Cad-6* may have a more direct role in the efficient fusion of mesenchyme-derived and ureteric bud-derived epithelium. In both mesenchymal aggregates and comma-shaped bodies, the *Cad-6*-expressing epithelium is directly abutted to the E-cadherin-expressing cells (Cho *et*

compared to wild-type animals of approximately the same age. Data are the averages from three mutant animals and three wild-type animals (10–20 random sections from each kidney) $\pm$ SD ($P < 0.001$, Student's *t* test). This number may represent a more accurate measure of the total number of necrotic glomeruli and tubules which are lost due to the *Cad-6* mutation. There was no significant difference between the numbers of necrotic glomeruli and tubules in E18.5 *Cad-6* mutant kidneys compared to control littermates ($P < 0.5$, Student's *t* test, data not shown). This suggests that necrosis is occurring shortly after birth and correlates with the onset of kidney function.

al., 1998). In fact, Cad-6 expression is highest in those tubule cells between the R-cadherin and the E-cadherin epithelium. Perhaps expression of the type II cadherin Cad-6 promotes efficient fusion of two disparate cell types that express different type I cadherins. Evidence for cadherin function during tubular epithelium fusion also comes from analysis of the *shotgun* gene (*shg*), which encodes *Drosophila* E-cadherin (DE-cadherin). Intermediate loss of function of *shg* results in failure of the branched tubular epithelia of the trachea to fuse and form a contiguous respiratory network (Uemura et al., 1996; Tanaka-Matakatsu et al., 1996). DE-cadherin accumulates at the sites of tracheal branch fusion and is thought to mediate the epithelial reorganization that occurs when the branches fuse (Tanaka-Matakatsu et al., 1996).

The Role of Signaling in Epithelial Fusion

Another possible reason for the fusion defect we observe is that due to the epithelialization delay, signaling mechanisms which regulate the fusion event may become asynchronous. Similarly, it is tempting to ask whether the expression of multiple cadherin subtypes in the early mesenchymally derived tubules is a mechanism for the regulation of β -catenin signaling. Indeed, it has been shown that overexpression of cadherins or underexpression of β -catenin both lead to an inhibition of dorsal mesoderm induction, consistent with the hypothesis that the regulation of free β -catenin is important for proper signaling (Heasman et al., 1994). In a like manner, overexpression of full-length DE-cadherin or just the cytoplasmic and transmembrane domains can mimic *wingless* phenotypes, suggesting that regulation of levels of cadherin expression can titrate the amount of β -catenin that is available for signaling pathways (Sanson et al., 1996). Conversely, there is evidence that the modulation of Wnt-1 signaling can affect cell-cell adhesion (Bradley et al., 1993). An attractive hypothesis to explain our observations is that R-cadherin alone can mediate the correct expression of junctional proteins and can mediate the formation of the early adherens junctions in the renal vesicle and drive the mesenchymal-epithelial transition. Furthermore, the hypothesis suggests that the expression of Cad-6 may serve as a mechanism to regulate the amount of β -catenin available for signaling which may control late aspects of epithelialization, such as basement membrane deposition and/or delamination and fusion to the ureteric bud. The analysis of R-cadherin mutant mice or mice harboring mutations in both Cad-6 and R-cadherin will be necessary to fully characterize the potential compensatory and/or combinatorial mechanisms which underlie the mesenchyme-epithelial transition during nephrogenesis.

We report here the targeted mutation and analysis of a type II cadherin, Cad-6. Cad-6 is involved in the efficient transition of induced mesenchyme to fully polarized epithelium, as mutant mice show a marked delay in formation of laminin-positive, mesenchymally derived structures both *in vivo* and *in vitro*. This delay significantly affects

fusion of the mesenchyme-derived, comma-shaped body to the ureteric bud epithelium. The resulting kidneys exhibit fewer nephrons postnatally due to necrosis of apparently blocked tubules. Such defects demonstrate the importance of timing during the critical events of tubule development in the kidney.

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Cadherin-6, a Cell Adhesion Molecule Specifically Expressed in the Proximal Renal Tubule and Renal Cell Carcinoma

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ABSTRACT

Cadherins are a family of calcium-dependent, cell-cell adhesion molecules that play an important morphoregulatory role in a wide variety of tissues. Alterations in cadherin function have been implicated in tumor progression in a number of adenocarcinomas. Despite the increasing number of new cadherins identified, little is known about cadherins in normal renal tissue and renal carcinomas. A novel cadherin transcript, cadherin-6, was recently described to be present in renal cancer cell lines and fetal kidney, but no data on protein expression nor tissue localization has been reported.

In this study, we demonstrate that the expression of cadherin-6 is restricted to the proximal tubule epithelium. This finding is critical because these cells give rise to the majority of neoplasms of this organ. Furthermore we demonstrate typical cadherin features of cadherin-6, including cytoplasmic binding to α - and β -catenin. We present data of cadherin-6 expression in a series of 32 primary renal cell cancers. Cadherin-6 expression tended to vary with histology in these samples. Whereas the majority of renal cell cancers with histology-associated poor prognosis (*i.e.*, high grade clear cell carcinomas and sarcomatoid renal tumors) show aberrant expression of cadherin-6, in tumors with a favorable prognosis (*i.e.*, low grade clear cell carcinomas and papillary cancers), normal cadherin-6 expression was predominant. Overall, these findings demonstrate specific expression of cadherin-6 in the proximal renal tubules in normal human kidney and suggest that alterations of cadherin-6 expression are associated with progression of renal cell carcinoma.

INTRODUCTION

Renal cell cancer accounts for approximately 3% of all adult malignancies. Surgical treatment is an effective therapy only in early-stage renal tumors. Advanced stages of renal neoplasms are associated with poor prognoses, because these malignancies are typically refractory to chemo- and radiotherapy. Immunotherapy, both active and passive protocols, demonstrate partial and complete remission in a limited subset of patients (1, 2). Prognostic markers are needed to identify patients who are at risk for recurrence and metastasis of renal cell carcinoma and, thus, would benefit from additional therapeutic modalities. Current prognostic factors like tumor grade, renal vein involvement, and extension to regional lymph nodes are of limited value in this respect (3).

Cadherins, a family of cell-cell adhesion molecules, have demonstrated their utility as prognostic markers in a variety of neoplasms. Dysfunction of cadherins has a major impact in the progression of epithelial tumors (4). Loss or abnormal expression of cadherins in tumors can lead to tumor invasion and disease progression, as demonstrated for E-cadherin expression in prostate (5), stomach (6),

bladder (7), breast (8), cervical cancer (9), and other tumors. Similar results have been demonstrated for P-cadherin, for example in breast cancer (8), gingival carcinomas (10), and gastric neoplasms (6).

Studies of the role of abnormal cadherin expression as a marker in renal cell carcinoma progression are less conclusive. Although the proximal tubular cell is thought to be the cellular origin of the majority of renal cell carcinomas, the pattern of cadherin expression in the normal proximal tubule epithelium is not well understood (11-13). Interestingly, for E-cadherin, which has been shown to be expressed in a high proportion of low grade renal carcinomas (14), the expression pattern failed to correlate with disease progression (15, 16). Normally, E-cadherin expression is restricted to the distal renal tubules and collecting ducts (17). N-cadherin, a cell adhesion molecule found in a variety of different tissues, is expressed during fetal development in renal tissue and is found in the adult kidney only in the proximal renal tubule (18). However, N-cadherin expression in renal cell cancer also failed to show a correlation with tumor progression. Tani *et al.* (16) suggested that another unknown cadherin may be important in these tumors.

Shimoyama *et al.* (19) reported that cadherin-6, a newly identified cadherin molecule, is involved in the development of the kidney. In adult renal tissue, however, only faint or negligible expression of cadherin-6 mRNA could be demonstrated (19, 20). Neither protein expression nor tissue localization of cadherin-6 was examined in these studies. Shimazui *et al.* (21) reported a complex pattern of cadherin mRNA expression in different renal cancer cell lines. This finding suggested the involvement of several cadherins in renal tumors (21).

In this study, we focused upon cadherins that may play a role in the cell-cell adhesion in the proximal renal tubular epithelium and, therefore, possibly may be involved in invasion and progression of renal cell cancer. We demonstrate for the first time that cadherin-6 expression occurs only in proximal renal tubules of the normal kidney. Furthermore, in an evaluation of a series of primary renal cell carcinomas, we find that cadherin-6 expression is frequently altered in tumors with histologies associated with poor prognosis.

MATERIALS AND METHODS

Surgical Specimens. Fresh renal tissue was obtained at the time of surgery at The Johns Hopkins Hospital. The specimens were immediately frozen in liquid nitrogen and stored at -70°C . Frozen sections were cut at a thickness of 6 μm , mounted on poly-L-lysine-coated slides, air dried for 30 min, and used immediately for immunohistochemistry.

Renal Cancer Cell Lines. Human primary renal cancer cell lines from the ATCC³ were used. 786-O and 769-P were cultured in RPMI 1640 supplemented with 10 mM HEPES buffer, 5 mM sodium pyruvate, and 10% FBS. McCoy's 5a medium with 10% FBS was used as medium for Caki-1 and Caki-2. Primary renal cancer cell lines were derived from surgical specimens by digestion with collagenase (2 mg/ml in HBSS with calcium and magnesium). RCC74 originated from a papillary renal cell carcinoma, stage II, nuclear grade III-IV. RCC71A was derived from a sarcomatoid renal cancer, stage II, nuclear grade IV. RCC 71A and RCC74 did not show any expression of E- and

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³ The abbreviations used are: ATCC, American Type Culture Collection; FBS, fetal bovine serum.

P-cadherin on immunohistochemistry and only faint expression of N-cadherin. NK-1 is a proximal renal tubule cell strain derived from a normal kidney. Primary renal cell lines were cultured in DMEM, supplemented with 10% FBS, L-glutamine, penicillin, streptomycin, and tryptose phosphate as described previously (22).

Northern Analysis. For Northern analysis of cadherin-6 mRNA, a random prime-labeled sequence corresponding to nucleotides from position 2108 to 2323, according to the cDNA sequence published by Shimoyama *et al.* (19) was used as a probe as described previously (23). Multitissue blots [2 μ g of poly(A) mRNA each] obtained from Clontech Laboratories (Palo Alto, CA) were probed using the methodology described previously (24).

Antibodies. A rabbit polyclonal anti-cadherin-6 antibody was generated against a sequence in the intracytoplasmic domain of cadherin-6 as described (25). This 15-mer (NH₂ terminal-RRTPARDNTDVRDF-COOH terminal) is unique by search at the National Center for Biotechnology Information using BLAST network service (26) for cadherin-6 in humans and K-cadherin in rats. Anti-E-cadherin clone 36 is a mouse monoclonal antibody (Transduction Laboratories, Lexington, KY) described previously (27). 13A9 and IG5 are mouse monoclonal antibodies against N-cadherin and α -catenin, respectively (28). Anti- β -catenin clone 14 is a mouse monoclonal antibody (Transduction Laboratories), which is described elsewhere (29). F23 (a kind gift from Dr. Neil H. Bander, Memorial Sloan-Kettering Cancer Center, New York, NY) is a mouse monoclonal antibody specific for proximal renal tubules (30). CY-90 is a mouse monoclonal anti-cytokeratin 18 antibody (Sigma Immuno Chemicals, St. Louis, MO; Ref. 31).

Immunohistochemistry. Immunohistochemistry was performed at room temperature unless mentioned otherwise using the Histostain-Kit (Zymed Laboratories, Inc., San Francisco, CA). Air dried sections were fixed in 3.7% formaldehyde in TNC buffer (10 mM Tris, 150 mM NaCl, and 2 mM CaCl₂, pH 7.4) for 20 min. Extraction was carried out prior to fixation for 10 min in TNC buffer containing 2% Triton X-100 and the protease inhibitors leupeptin (50 μ g/ml) and phenylmethylsulfonyl fluoride (10 mM) if the sections were to be used for cadherin-6, E- or N-cadherin staining. Between each step, rinsing was carried out using TNC buffer three times for 2 min. Sections were blocked with 10% nonimmune goat serum for 30 min. The primary antibody was applied for an incubation period of 1 h (dilution of antisera in 3.75% BSA and TNC buffer: 1:1000 for cadherin-6, 0.5 μ g/ml for E-cadherin and β -catenin, 1:50 for N-cadherin conditioned media, 1:100 for α -catenin conditioned media, and 1:1000 for F23). After rinsing, specimens were incubated with either biotinylated goat anti-rabbit-IgG (cadherin-6) or goat anti-mouse-IgG, followed by a 15-min step of conjugation with streptavidin-peroxidase complex. Staining was developed with aminoethyl carbazole chromagen substrate in 0.006% peroxide containing buffer within 30 min, followed by rinsing with tap water (three times). Finally, the sections were counterstained with hematoxylin, dehydrated, and mounted. Rabbit preimmune serum was used as a control for cadherin-6 staining. To demonstrate the cadherin-6 antibody specificity, blocking assays were performed with the synthetic peptide used for immunization present at concentrations up to 1 mg/ml. Even up to 1 mg/ml the peptide had no effect on staining with the E-cadherin antibody.

Immunofluorescence. Cells were grown to near confluence on two-chamber glass slides. Cells and sections were fixed first and extracted as described for immunohistochemistry. Rinsing was carried out between these steps with TNC buffer (three times for 2 min each). Then specimens were treated with BSA 3.75% in TNC buffer for 1 h before incubating with the primary antibody overnight. Both steps were carried out at 4°C. After rinsing the secondary antibody-fluorescein isothiocyanate-conjugated AffiniPure donkey anti-rabbit IgG or tetramethylrhodamine isothiocyanate-conjugated AffiniPure donkey anti-mouse IgG (both from Jackson Immuno Research Laboratories, Inc., West Grove, PA) were applied and incubated at 4°C for 1 h. Nuclear counterstaining was performed by adding 4',6-diamidino-2-phenylindole (0.25 μ g/ml) to the secondary antibody. Slides were treated with SlowFade antifade kit (Molecular Probes, Inc., Eugene, OR) and mounted with coverslips.

Immunoprecipitation. Cells were grown in a 6-cm culture dish (Falcon, Lincoln Park, NJ) until about 50% confluent. At that time, cells were labeled with [³⁵S]methionine (Amersham Life Science, Arlington Heights, IL) at a concentration of 200 μ Ci/ml in methionine-free medium for 16 h (37°C, 5% CO₂, humidified atmosphere). After two washes in HBSS, cells were extracted in TNC with 2% Triton X-100, 10 mM phenylmethylsulfonyl fluoride, and 50 μ g/ml leupeptin for 5 min at 4°C. Cells were scraped, homogenized (10 strokes

in a steel dounce), and centrifuged for 50 min at 100,000 $\times$ g at 4°C. Two hundred μ l of the supernatant were incubated with either cadherin-6 (2 μ l), α -catenin (100 μ l), β -catenin (1 μ g), or culture medium as a control overnight on ice. Immunocomplexes were captured by Protein G-Plus/Protein A agarose beads (Oncogene Science, Cambridge, MA) and washed twice in extraction buffer. Immunocomplexes were eluted from capture resin in SDS sample buffer (2% SDS, 1.25% β -mercaptoethanol) and separated by 10% SDS-PAGE (0.75-mm gels). The gel was stained and destained as described (24). Autoradiographs were exposed at -70°C. For immunoprecipitation experiments, cells were grown in T75 culture flasks and processed as above, except radioactive labeling was omitted.

Immunoblotting. Frozen renal tissue stored at -70°C or 80% confluent cell cultures were lysed in 2% SDS, 100 mM NaCl, 1 mM EDTA, and 62.5 mM Tris-HCl, pH 6.8. The protein extract was centrifuged for 20 min at 14,000 rpm at 4°C. Supernatant was retained, and total protein concentration was calculated by BCA Protein Assay Reagent A (Pierce Immunochemicals, Rockford, IL). Fifty μ g of total protein in a final solution of 2% SDS, 1.25% β -mercaptoethanol, 1 mM EDTA, and 62.5 mM Tris-HCl (pH 6.8) were separated on 10% SDS-PAGE (1.5-mm gel) and transferred to nitrocellulose membrane (Hybond-ECL, Amersham Life Science, Arlington Heights, IL) in 20% methanol and 0.05% SDS. Nitrocellulose membranes were blocked in 5% nonfat dry milk and Tris-saline buffer overnight. Immunoblotting was performed with the primary antibody at a dilution of 1:10,000 for cadherin-6 and 0.5 μ g/ml for β -catenin for 3 h at room temperature in Tris-saline buffer with 0.1% nonfat dry milk. As a secondary antibody, horseradish peroxidase-conjugated IgG (donkey anti-rabbit or sheep anti-mouse, 1:10,000; Amersham Life Science) was used in Tris-saline buffer with 5% nonfat dry milk. Before and after this step, immunoblots were washed three times each for 20 min in Tris-saline buffer with 1% Tween 20. Chemiluminescence was developed with Super Signal solution (Pierce Immunochemicals, Rockford, IL).

RESULTS

Cadherin-6 mRNA Is Restricted to the Kidney. In an effort to characterize cadherin expression in a neonatal prostate epithelial cell line, we cloned a novel cadherin cDNA using degenerate PCR technology (32). A homology search revealed that the sequence of this cadherin was identical to that of cadherin-6, recently characterized by Shimoyama *et al.* (19) to be present in some renal carcinoma lines and in fetal kidney tissue. The localization of expression of cadherin-6 in the normal kidney as well as the possible alterations of cadherin-6 expression in renal cell cancer was not determined in this previous study.

By Northern analysis we confirmed the tissue specificity of cadherin-6, because the analysis of 16 different adult tissues demonstrated detectable mRNA message only for kidney. In this tissue, three transcripts of about 3.6, 4.0, and 8 kb were observed (Fig. 1A). All other tissues, including normal adult prostate, were negative.

Characterization of the Cadherin-6 Antibody. To more fully characterize the expression pattern of cadherin-6 in both normal and neoplastic renal tissues, we developed a polyclonal antiserum against cadherin-6. A unique peptide sequence located in the cytoplasmic domain of cadherin-6 was used as the antigen.

The specificity of this antibody was assessed by immunoblotting and immunofluorescence with and without blocking peptide. The former analysis revealed the presence of a single band of 120 kDa (Fig. 1B), the size of a typical cadherin, in the renal cancer cell line RCC74. Furthermore, the lack of reactivity of this antiserum with E- or P-cadherin was demonstrated by immunoblot analysis of cell lines known to be positive for these cadherins (data not shown).

Immunofluorescent staining of RCC74 cells with the cadherin-6 antiserum demonstrated immunoreactivity at the cell-cell border (Fig. 1C), whereas no staining was observed using preimmune rabbit serum. To demonstrate the specificity of our antibody for cadherin-6, the synthetic peptide used as antigen was added to the primary antibody at different concentrations up to 1 mg/ml. In contrast to the

CADHERIN-6 IN RENAL CELL CARCINOMA

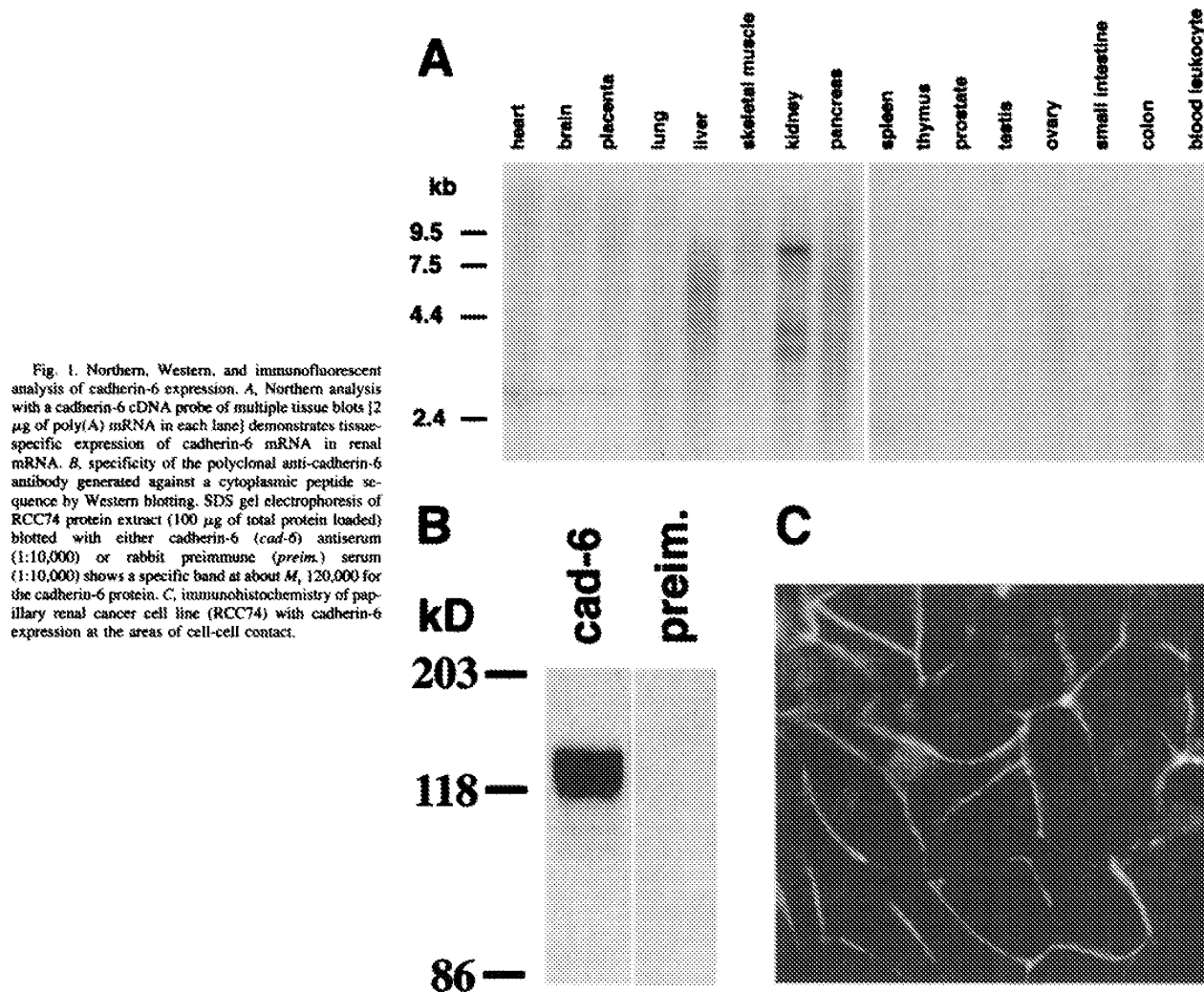


Fig. 1. Northern, Western, and immunofluorescent analysis of cadherin-6 expression. **A**, Northern analysis with a cadherin-6 cDNA probe of multiple tissue blots [2 μ g of poly(A) mRNA in each lane] demonstrates tissue-specific expression of cadherin-6 mRNA in renal mRNA. **B**, specificity of the polyclonal anti-cadherin-6 antibody generated against a cytoplasmic peptide sequence by Western blotting. SDS gel electrophoresis of RCC74 protein extract (100 μ g of total protein loaded) blotted with either cadherin-6 (*cad-6*) antiserum (1:10,000) or rabbit preimmune (*preim.*) serum (1:10,000) shows a specific band at about M_r 120,000 for the cadherin-6 protein. **C**, immunohistochemistry of papillary renal cancer cell line (RCC74) with cadherin-6 expression at the areas of cell-cell contact.

positive staining of normal kidney without the peptide, there was no staining observed at concentrations as low as 0.01 mg/ml. To exclude nonspecific effects, the same peptide was added to anti-E-cadherin antibody, followed by immunohistochemical staining. Under these conditions, staining was unaffected (data not shown).

Expression of Cadherin-6 in the Proximal Tubule of the Normal Kidney. We established a cell culture of proximal renal tubular epithelium (NK-1), which showed positive staining of F23 and cytokeratin 18, indicating its origin from the epithelium of the proximal renal tubule (data not shown). With cadherin-6 antiserum, NK-1 cells showed positive immunoreaction at the cell-cell border in typical cadherin-like pattern at areas of cell-cell borders but not at cell membranes without cell-cell contact (Fig. 2a).

On frozen sections of normal renal tissue, we could demonstrate staining for cadherin-6 in the renal tubular epithelium at the cell-cell borders (Fig. 2b). To further characterize this staining in the renal tubular epithelium, serial sections of normal renal tissue were prepared from surgical specimens and immunohistochemically stained for cadherin-6, F23, and E-cadherin as shown in Fig. 2, c–e. The staining of cadherin-6 in the renal cortex (Fig. 2d) shows the same distribution as the staining for the membranous glycoprotein F23 (Fig. 2c), which is specific for the proximal renal tubules (30). The same

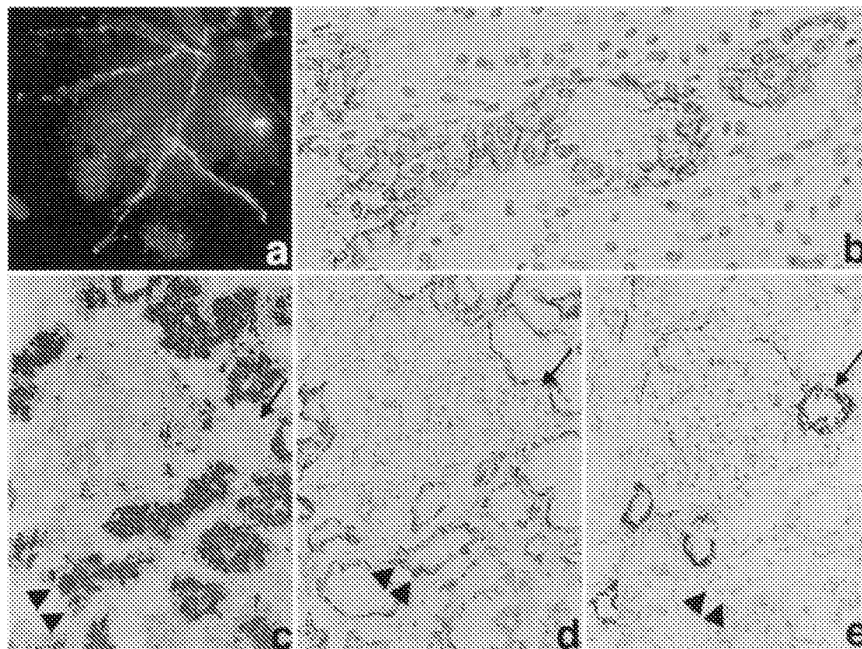
tubules showed positive staining for N-cadherin (data not shown). In contrast, E-cadherin expression was observed in F23-negative tubules, including the distal renal tubules and collecting ducts (Fig. 2e). No staining of distal tubules and collecting ducts could be observed for cadherin-6, and as expected, proximal tubules were negative for E-cadherin. Except for some scattered positive areas in the Bowman's capsule for all three antibodies used, no cadherin expression could be identified within the glomeruli. The specificity of cadherin-6 positivity in the proximal renal tubule, according to F23-positive tubules, was confirmed by immunofluorescence (data not shown). E-cadherin again was restricted to F23-negative distal renal tubules.

On higher power, cadherin-6 immunofluorescence (Fig. 3a) appears not to be limited to the areas of cell-cell contact but also is present in the areas of the basal membrane. This finding correlates with the localization of the coexpressed α -catenin (data not shown) and β -catenin (Fig. 3b). In contrast, E-cadherin-positive (Fig. 3c) and N-cadherin-positive (Fig. 3d) tubular cells, which also demonstrate colocalization with α - and β -catenin (data not shown), do not show an enhancement of staining at the basal membrane.

Interaction of Cadherin-6 with the Catenins. The classical cadherins are known to interact with a family of cytoplasmic proteins termed catenins. Interaction of cadherins with these proteins is a

CADHERIN-6 IN RENAL CELL CARCINOMA

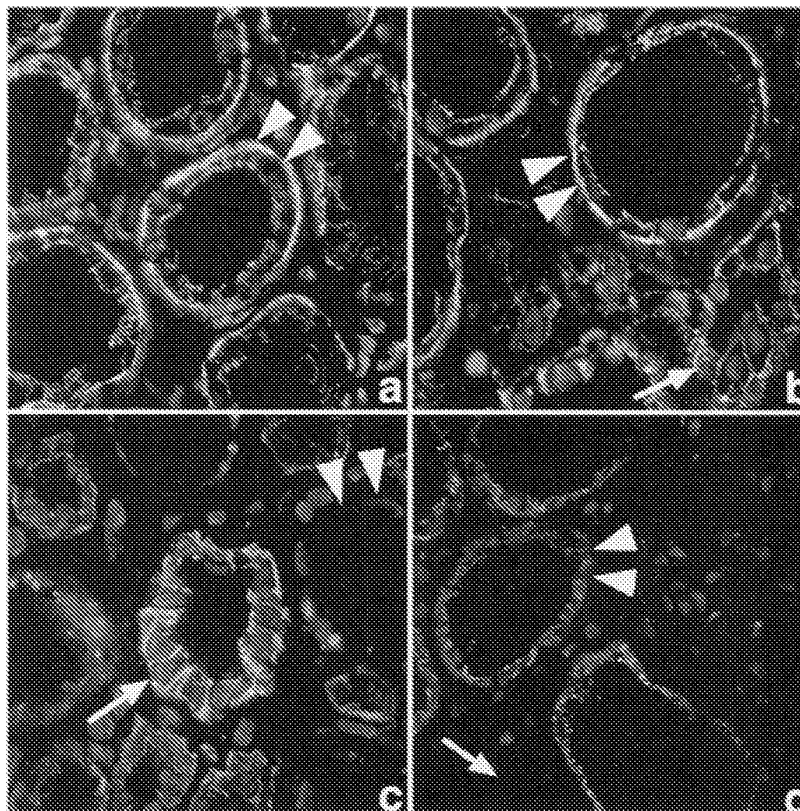
Fig. 2. Immunostaining of cadherin-6 in normal human kidney. *a*, cadherin-6 immunoreactivity in NK-1 cells, a strain of normal proximal tubular renal cells. The staining of cadherin-6 is located at the cell borders in areas of cell-cell contact; no staining is observed at cell membranes without cell-cell contact. *b-e*, frozen sections of normal kidney. *b*, cadherin-6 staining at the cell-cell border of the renal tubular epithelium. Note honeycomb staining pattern. *c-e*, immunostaining of serial sections of normal renal tissue. In these panels, the two arrowheads indicate the same proximal renal tubule, and the arrows show the identical distal renal tubule in each section surrounding a glomerulus. *c*, F23 (1:1000), an antibody specific for the proximal renal tubules. *d*, cadherin-6 antiserum (1:1000) showing positive staining of tubules having an identical distribution to that of F23. *e*, anti-E-cadherin (1 μ g/ml) expression in the distal renal tubules. *a*, $\times 1000$; *b-e*, $\times 400$.



prerequisite for normal cadherin function (33). To investigate the potential interaction of cadherin-6 with these proteins, we performed immunoprecipitation and immunoconcentration experiments. The autoradiograph of cadherin-6 immunoprecipitates from metabolically labeled RCC74 cells (Fig. 4A) demonstrates coprecipitation of pro-

teins with the apparent molecular weights (M_r 102,000 and M_r 92,000) corresponding to that of α - and β -catenin. To determine the identity of the coprecipitating species as catenins, we carried out Western blotting of immunoprecipitates obtained with cadherin-6 and β -catenin antibodies (Fig. 4B). We immunoprecipitated with either cad-

Fig. 3. Immunofluorescent staining of sections of normal human kidney with fluorescein-conjugated secondary antibody and nuclear counterstaining with 4',6-diamidino-2-phenylindole. *a*, cadherin-6 immunoreactivity of positive proximal renal tubules at the areas of cell-cell contact and enhanced staining at the basal membrane (arrowheads). *b*, β -catenin is localized in the proximal (arrowheads) and distal renal tubules (arrow). Note the observed enhancement of the basal membrane in proximal renal tubules identical to the localization of cadherin-6 expression. *c*, E-cadherin-positive distal renal tubules (arrow) without enhanced staining at the basal membrane; proximal renal tubules show no staining (arrowheads). *d*, N-cadherin-positive proximal renal tubules (arrowheads). Note N-cadherin-negative distal renal tubules (arrow). $\times 400$.



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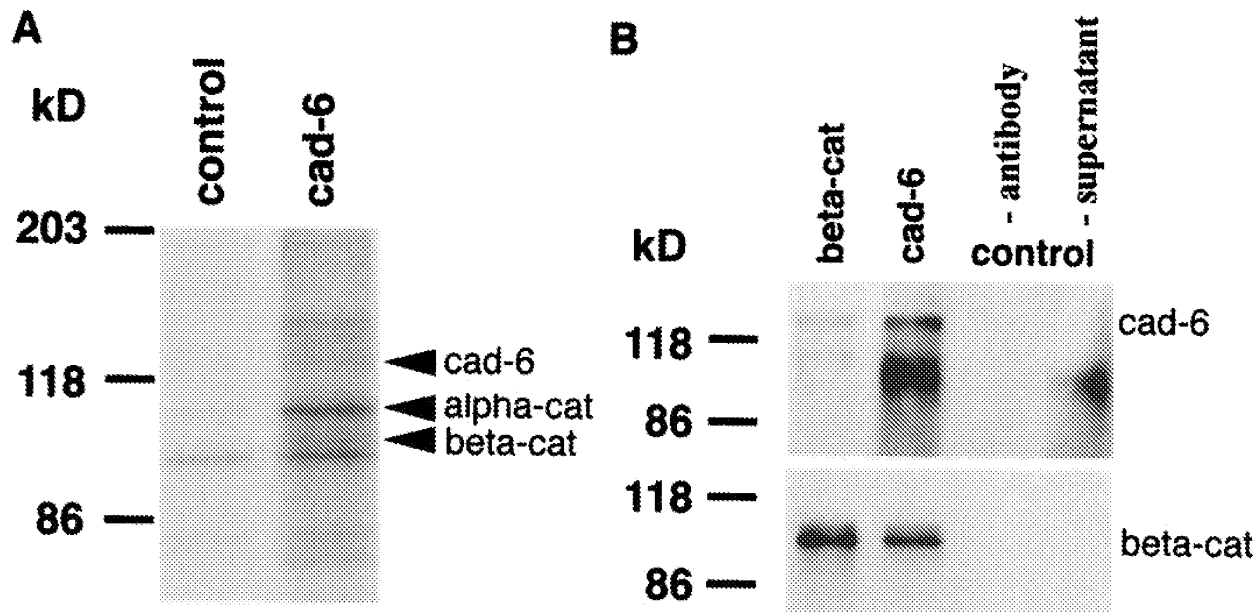


Fig. 4. Immunoblot and immunoprecipitation analysis to determine cadherin-6 expression and interaction with intracytoplasmic proteins. A, autoradiograph of cadherin-6 immunoprecipitated RCC74 cell extracts demonstrating coprecipitating proteins of apparent molecular weight according to α -catenin (M_r 102,000) and β -catenin (M_r 95,000). As control, tissue culture medium was incubated with the labeled cell extract instead of cadherin-6 antiserum. B, immunoblot of β -catenin and cadherin-6 immunoprecipitates from cell extracts of RCC74. The upper gel was probed with anti-cadherin-6 antiserum, and the lower gel was probed with anti- β -catenin. The first lane of the upper gel demonstrates the recovery of cadherin-6 using β -catenin antibodies, and the second lane of the lower gel demonstrates the recovery of β -catenin with cadherin-6 antiserum, confirming immunoprecipitation results presented in A. As control, cell extract without antibody (-antibody) and tissue culture medium incubated with cadherin-6 antibody (-supernatant) were used.

herin-6 or β -catenin antibody, and by subsequent Western blotting of these immunoprecipitates with either cadherin-6 or β -catenin antibodies, we found coprecipitation of β -catenin with cadherin-6 antiserum, and likewise, cadherin-6 coprecipitated with β -catenin antibody, indicating their binding interaction in the cell extracts. As negative controls, we used cell extracts immunoprecipitated with nonimmune tissue culture medium prior to Western blotting, as well as substituting culture medium for the cell extract during the immunoprecipitation step (Fig. 4B).

Cadherin-6 Expression in Renal Cancer Cell Lines and Primary Renal Cell Carcinomas. We have established two primary renal cancer cell lines from tumors of different histological type. RCC74 is from a papillary renal carcinoma and shows intense expression of cadherin-6, and RCC71A is derived from a sarcomatoid renal cell cancer and proved to be cadherin-6 negative. Human renal carcinomas obtained from the ATCC showed different levels of cadherin-6 expression. 769-P and 786-O showed only very weak staining at the cell-cell borders, but 786-O demonstrated diffuse cytoplasmic staining, as did Caki-1 and Caki-2 (data not shown).

To examine cadherin-6 expression in primary samples of renal cell cancer, we examined frozen sections for the expression for cadherin-6 and E-cadherin by immunohistochemistry. The samples were scored as either negative (Fig. 5a), positive (Fig. 5b), or heterogeneous (Fig. 5c). We looked at a total of 32 renal cell cancers stratified by the histological type and tumor grade (Table 1). Tumors with clear cell features demonstrated cadherin-6 retention in low-grade tumors and heterogeneous or loss of expression in high-grade tumors. All papillary tumors revealed strong cadherin-6 positivity, whereas sarcomatoid tumors showed loss of cadherin-6 expression. Oncocytomas, which are thought to arise from the collecting duct system (34), proved to be E-cadherin positive and cadherin-6 negative, as would be expected from our observations in normal kidney.

To confirm the results of the immunohistochemistry, Western anal-

yses were performed. According to the results of the immunofluorescence experiments, RCC74 demonstrated a strong signal for cadherin-6, whereas RCC71A showed no immunoreactivity (Fig. 6A). No protein signal could be detected for Caki-1 and only very faint expression for Caki-2 and 769-P. Of the ATCC cell lines, only 786-O showed abundant cadherin-6 expression; besides the band at M_r 120,000, several smaller proteins were detected, presumably due to degradation or abnormal products (Fig. 6A). Attempts to minimize protein degradation by direct solubilization of cultured cells in SDS sample buffer resulted in no change in the pattern of immunoreactive bands. Similar findings have been reported for Ksp-cadherin in the rabbit kidney (35), although the origin of these smaller polypeptides remains unclear.

Three matched pairs of normal renal tissue and renal cell cancer (Fig. 6B) were analyzed demonstrating either up-regulation (sample 140), equal expression (sample 246), or no expression (sample 273) of cadherin-6 in renal tumors compared to normal. On histological examination of these tumors, the two cadherin-6-positive tumors revealed clear cell features, whereas the cadherin-6-negative tumor was an oncocytoma.

DISCUSSION

In this study, we demonstrate that cadherin-6 is specifically expressed in the proximal renal tubule in human kidney. Cadherin-6 shows two features typical of classical cadherins: (a) staining at the cell-cell border at the areas of cell-cell contact; and (b) interaction with the cytoplasmic proteins α - and β -catenin. Furthermore, we examined a series of primary renal cell cancers, finding that loss of cadherin-6 expression is more common in high-grade tumors or tumors with worse prognosis. These observations implicate a possible role of cadherin-6 as an important factor in renal tumor progression.

To understand the development and progression of a given cancer

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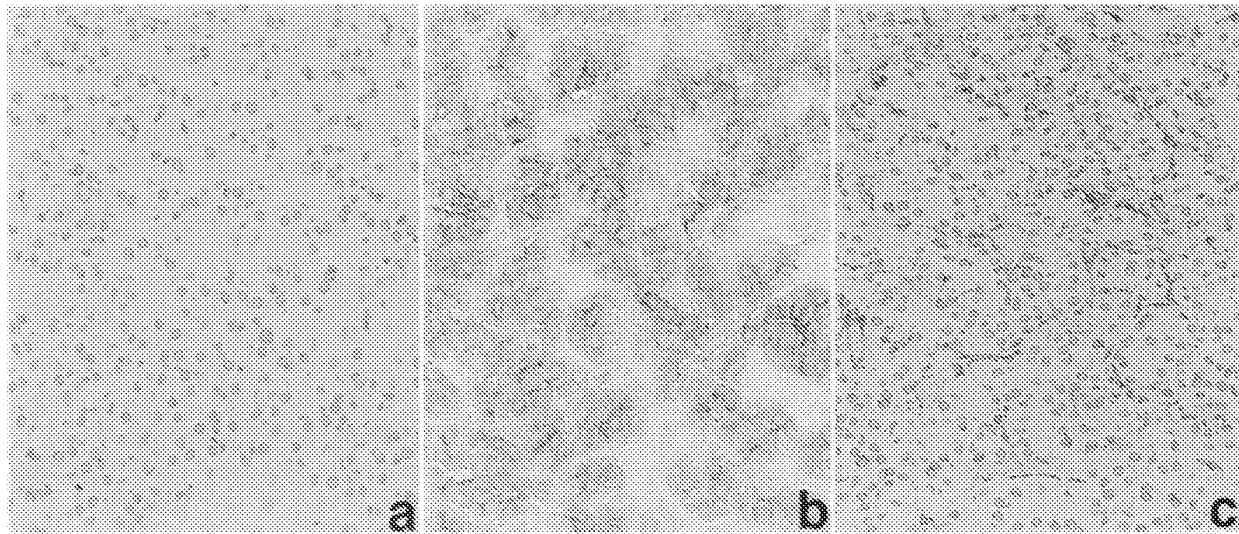


Fig. 5. Immunohistochemistry of sections of renal cell carcinomas with cadherin-6 antiserum (1:1000). a, lack of cadherin-6 expression in an oncocytoma. b, retained expression at cell-cell borders in renal cancer with clear cell features. c, down-regulation and heterogeneous expression of cadherin-6 in a clear cell carcinoma. $\times 400$.

type, it is necessary to investigate the normal architecture and function of the cells that are destined to be transformed. One important aspect of carcinogenesis involves cell adhesion molecules as possible factors in tumor invasion, tumor cell circulation, and metastatic implantation. Cadherins are transmembrane glycoproteins that interact in a calcium-dependent, homotypic fashion. Besides the well known classical cadherins epithelial (E-), neural (N-), and placental (P-) cadherin, more than 30 additional cadherins have been described. Cadherins play an important role in tissue compaction and cell differentiation, maintenance of tissue, cell migration, and cell regulation (36). Cadherins are also involved in tumor aggressiveness and tumor invasion (37). In a variety of epithelial neoplasms, loss of cadherin expression correlates with tumor progression. Cadherins are thought to act as tumor invasion suppressor genes, as demonstrated by E-cadherin in gastric and colorectal carcinomas (4), breast cancer (8), skin neoplasm (38), transitional cell carcinoma of the bladder (7), and prostate cancer (24, 39). However, in renal cell cancer, no cadherin molecule correlating with either prognosis or invasion has been identified (15, 16).

Terpe *et al.* (14) demonstrated that E-cadherin expression can be found in about 30% of all renal tumors and is restricted to low-grade cancers; they suggested that E-cadherin expression may be connected to a dedifferentiation process. Katagiri *et al.* (15) also investigated E-cadherin expression in renal tumors. They found that none of the metastasis expressed E-cadherin, but about 20% of the primary lesions showed positive immunoreactivity. The authors conclude that E-cadherin may be a prognostic marker in renal cell cancer, but because

of the low rate of expression, other cell adhesion molecules may play a more important role. N-cadherin, also called A-CAM, which is known to be expressed during the renal development and in the proximal renal tubules of the adult kidney (40), has also been investigated. Tani *et al.* (16) reported that E- and N-cadherin expression in renal cell cancer does not correlate with tumor grade. They concluded that aberrations in another pathway must be responsible for the deficient capacity to form polarized tubular structures in kidney cancer. A novel cadherin molecule, cadherin-6, was isolated by Shimoyama *et al.* (19) from a hepatocellular carcinoma lacking E- and P-cadherin expression. This cadherin showed 97% homology with the cDNA of K-cadherin, which was cloned by Xiang *et al.* (20) from a rat renal cancer cell line. K-cadherin could not be detected in adult rat kidneys, but RNA transcripts of human cadherin-6 were found by Northern analysis of adult human kidneys and renal cancer cell lines (19). These first reports suggested that cadherin-6 is involved in the development of the kidney and possibly renal cell cancer.

Our results confirm the tissue specificity of cadherin-6 and establish for the first time that cadherin-6 is expressed in the proximal tubular epithelium. The distribution of cadherin-6 shows typical staining at the cell borders in areas of cell-cell contact *in vitro*. *In vivo* we observed that cadherin-6 is also localized at the basal membrane, along with β -catenin. We speculate that cadherin-6, which is specifically expressed in the proximal tubule, may contribute to the physiological function of these epithelial cells. Interestingly, Thomson *et al.* (35) describe a similar observation for Ksp-cadherin, which, like

Table 1 Expression of E-cadherin and cadherin-6 in primary renal cell carcinoma by immunohistochemistry

Renal cell carcinomas were stratified by histological type and tumor grade. Immunoreactivity was scored as positive (pos.), heterogeneous ($\pm$), or negative (neg.).

Tumor	Total	E-cadherin			Cadherin-6		
		pos.	$\pm$	neg.	pos.	$\pm$	neg.
Clear cell carcinoma							
Grade II	10	4	1	5	6	2	2
Grade III	8	1	3	4	1	4	3
Grade IV	2	0	1	1	0	1	1
Papillary renal cell carcinoma	4	0	1	3	4	0	0
Sarcomatoid renal cell carcinoma	3	0	1	2	0	2	1
Granular renal cell carcinoma	3	1	0	2	2	0	1
Oncocytoma	2	2	0	0	0	0	2
Total	32	8	7	17	13	9	10

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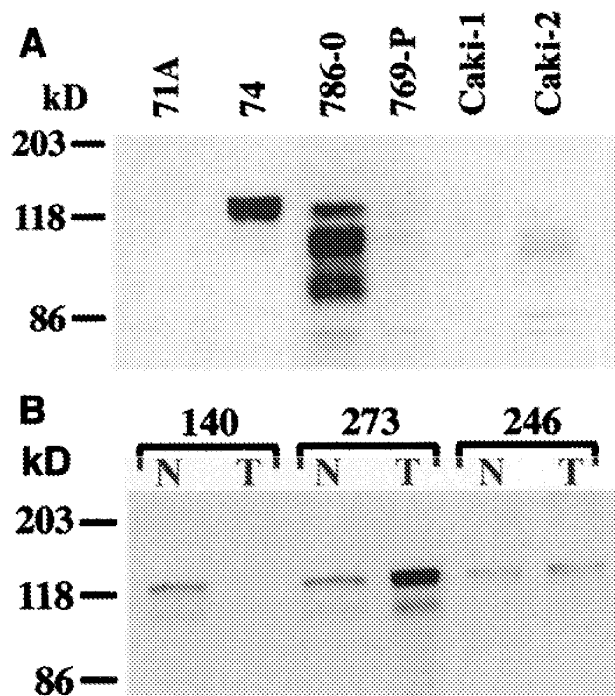


Fig. 6. Immunoblot analysis with cadherin-6 antibody (1:10,000) of renal carcinomas. A, renal cancer cell lines (100 μ g of total protein load per lane). Note smaller immunoreactive bands in cell line 786-O. B, matched pairs of normal kidney and renal carcinoma cell extracts. Tumors 246 and 273 were classified as clear cell carcinomas, whereas tumor 140 is an oncocytoma on histological examination.

cadherin-6 in humans, is a kidney-specific cell adhesion molecule in rabbits. In their report, immunofluorescence studies demonstrated that Ksp-cadherin is not limited to the lateral regions of cell-cell contact but showed a much broader basolateral distribution. Originally, their goal was to isolate a $\text{Na}^+/\text{HCO}_3^-$ cotransporter at the basolateral membrane, but the sequence of the transcript they identified showed homology to the cadherin family. It has recently been shown that E-cadherin and plakoglobin are involved in the formation of tight junctions (41). Xiang *et al.* (20) emphasize the possibility that cadherin function may vary from their well-established roles as cell adhesion molecules. Further studies will be necessary to clarify the function of basolaterally localized cadherin-6.

Because the majority of renal cell carcinomas are thought to arise from the proximal renal tubular epithelium (12, 42), a proximal tubular cadherin molecule could play an important role in renal cell tumorigenesis. Some studies demonstrate markers of both proximal and distal renal tubules to be present in renal cell carcinomas (43, 44), suggesting an origin from a metanephric cell rather than from a differentiated tubular renal cell. Our results demonstrate that the cadherin-6 protein is retained in some but not all renal tumors. We established two renal cancer cell lines from primary tumors of different histological type, a papillary renal cell carcinoma that retained cadherin-6 expression and a cadherin-6-negative sarcomatoid renal cancer. By immunohistochemistry, we demonstrated in 32 primary renal cell cancers that high-grade clear cell carcinomas are more likely to show heterogeneity or loss of cadherin-6 expression. Disease outcome varies for renal tumors of different histological type. Sarcomatoid tumors have a poor prognosis (43, 45), and these tumors demonstrated more heterogeneous cadherin-6 reactivity in our study. Papillary renal carcinomas demonstrated uniform positive staining. These tumors, which tend to have a more favorable prognosis (13),

have been suggested to originate from the collecting duct (46), although studies are contradictory regarding both these issues (47). Homogeneous staining for cadherin-6, however, is more consistent with the origin of these tumors from the proximal renal tubule and may explain the unusual papillary structure of polarized cells in contrast to solid clear cell carcinomas. However, the use of cell-specific markers to determine cell type of origin can be problematic, and it is certainly possible that acquisition of cadherin-6 expression accompanies cellular transformation in this tumor type. The renal tumor with the most favorable prognosis (48), the oncocytoma, is cadherin-6 negative, but this tumor has been shown to arise most likely from the collecting duct (34), and thus, cadherin-6 expression would not be expected.

In conclusion, we demonstrate that cadherin-6 is specifically expressed in the proximal renal tubules in humans. Cadherin-6 is retained in some but not all renal cell carcinomas. The observation of differential expression of cadherin-6 in renal cancers of different histological type and tumor grade and down-regulation of expression in a significant subset of renal cell carcinomas is consistent with an involvement of this cell adhesion molecule in the progression of renal cell cancer. Additional, direct studies of cadherin-6 dysfunction in renal cell carcinoma will be necessary to expand these findings and to fully elucidate the role of this process in renal cell cancer progression.

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Cadherin-6, a Cell Adhesion Molecule Specifically Expressed in the Proximal Renal Tubule and Renal Cell Carcinoma

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The Level of Cadherin-6 mRNA in Peripheral Blood Is Associated with the Site of Metastasis and with the Subsequent Occurrence of Metastases in Renal Cell Carcinoma

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BACKGROUND. To evaluate the significance of the presence of circulating renal cell carcinoma (RCC) cells in the development of metastases, the authors extended a previous study to quantify cadherin-6 mRNA levels in association with the pattern of metastasis.

METHODS. Cadherin-6 mRNA levels were measured in peripheral blood samples from 66 patients with RCC, including 55 patients who had newly diagnosed RCC (43 without metastases and 12 with metastases) and 11 patients who had recurrent RCC. For quantitative polymerase chain reaction analysis, a cutoff value was determined in blood samples from 25 healthy volunteers and was verified in samples from 5 healthy controls and from 10 patients who had other malignancies. The correlation between the site of metastases and the cadherin-6 mRNA level was analyzed, and a follow-up study (median, 39 months) to track subsequent metastases was performed after patients underwent nephrectomy.

RESULTS. Cadherin-6 was found in 69.9% of patients with metastases and in 34.9% of patients without apparent metastases ($P = 0.0099$). In the group of patients with recurrent RCC, patients who had only pulmonary metastases had a significantly lower positivity rate (25.0%) compared with patients who had distant metastases (85.7%; $P = 0.044$). Among 43 patients with newly diagnosed RCC, 5 of 15 patients who were positive for cadherin-6 had metastases after nephrectomy, whereas only 2 of the 28 patients with negative cadherin-6 status had recurrent disease ($P = 0.0398$). In addition, the recurrence-free survival of patients who were positive for cadherin-6 was poorer compared with the survival of patients who were negative for cadherin-6 ($P = 0.062$).

CONCLUSIONS. The quantification of cadherin-6 mRNA in peripheral blood may be a significant predictive marker for current and future metastases. However, subsequent metastases did not always correlate with levels of cadherin-6 mRNA. This may have been due either to the small numbers of circulating tumor cells or to the low levels cadherin-6 mRNA in circulating tumor cells. *Cancer* 2004;101:963-8.

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KEYWORDS: kidney, cell adhesion molecule, metastasis, prognosis, polymerase chain reaction.

Renal cell carcinoma (RCC) has an unpredictable clinical course and a tendency to recur and metastasize, even several years after surgery. Approximately 30% of patients with RCC already have metastatic disease at the time of diagnosis.¹ Although several new therapeutic modalities (e.g., cytotherapy, immunotherapy, and gene therapy) have been developed recently for the treatment of patients with

TABLE 1
Summary of Patients with Renal Cell Carcinoma and Quantification of Cadherin-6 mRNA

Characteristic	No. of patients	M/F	Tumor classification					Tumor grade				Cadherin-6 mRNA (relative ratio)	
			T1	T2	T3	T4	Tx	1	2	3	X	Median (range)	Average (SD)
Newly diagnosed RCC													
Without metastases (localized)	43	30/13	24	7	11	0	1	17	19	7	0	0.15 (0.003-1.25)	0.316 (0.404)
With metastases ^A	12	8/4	0	2	6	0	4	0	2	6	4	0.32 (0.135-3.31)	0.60 (0.069)
Recurrent RCC													
With metastases (post-Nx) ^B	11	8/3	0	4	5	1	1	---	---	---	---	0.345 (0.01-4.985)	0.817 (1.427)
Any RCC metastases (A + B)	23	16/7	---	---	---	---	---	---	---	---	---	0.325 (0.01-4.985)	0.704 (1.147)

M/F, male/female ratio; SD, standard deviation; RCC, renal cell carcinoma; meta, metastasis; Nx, nephrectomy.

metastatic RCCs, the prognosis for these patients remains poor. It is possible, however, that survival rates may be improved if these treatment modalities were carried out at an earlier stage in metastatic disease, i.e., during micrometastasis. From this point of view, a number of investigations have attempted to evaluate the malignant potential of RCC cells by detecting genetic alterations and trying to identify a molecular marker that is predictive of metastases.

Giroldi et al. showed that cell-cell adhesion molecules, the so-called cadherins, play an essential role in the preservation of normal tissue integrity and that their disruption correlates with the most initial phase of the metastatic cascade in many carcinomas, including urologic malignancies.² Cadherin-6, which is a Type 2 cadherin that probably is associated with tumor origin, is expressed frequently in RCC instead of E-cadherin.³ We previously reported that cadherin-6 had prognostic value, especially in patients with E-cadherin-absent RCC.⁴ Among patients with RCC who have abnormal cadherin-6 levels, survival rates are poorer statistically compared with the rates among patients with RCC who have normal cadherin-6 levels.⁴ In addition, we have noticed that mRNA expression of cadherin-6 often was increased in patients who had RCC with abnormal cadherin-6 levels⁵ and that this molecule also was expressed at the metastatic site (data not shown).

Recent investigations have revealed that micrometastasis can be predicted with polymerase chain reaction (PCR)-based molecular techniques using circulating blood.⁶⁻⁹ Although specific molecular markers for the detection of RCC cells in the peripheral blood have not been established, several basic investigations have been performed.^{5,10-12} In the current study, we extended our previous work,⁵ in which we attempted to detect circulating RCC cells by using a quantitative PCR method for the detection of cadherin-6 mRNA as a new molecular marker, and eval-

uated its clinical value for predicting micrometastasis and evaluating the metastasis-related biologic potential of RCC.

MATERIALS AND METHODS

Patients

Blood samples were collected from patients with RCC, including 55 patients with newly diagnosed RCC and 11 patients with recurrent RCC, after informed consent was obtained from each patient. Patients with recurrent RCC had undergone previous nephrectomy and had metastatic lesions. Among the 66 patients, the male:female ratio was 46:20. Among the 55 patients with newly diagnosed RCC, 43 patients had localized RCC without evidence of metastasis, and 12 patients had advanced disease with metastases (Table 1). Peripheral blood samples were collected from all patients with newly diagnosed RCC (55 patients) before nephrectomy and from 26 patients during the period 1-4 weeks after nephrectomy. In addition, peripheral blood samples were collected from patients at the time of diagnosis of recurrence with metastatic disease. The median follow-up after nephrectomy was 39 months (range, 30-60 months). Peripheral blood samples from 25 healthy volunteers were used as a control to determine the cutoff value of this assay. To verify the validity of the cutoff value, additional samples from 5 healthy controls and from 10 patients with other urologic malignancies (6 urothelial carcinomas, 3 prostate carcinomas, and 1 testicular carcinoma) also were analyzed.

RNA Extraction from Blood Samples and cDNA Synthesis

Blood samples were diluted with the same volume of phosphate buffered saline (PBS) and then layered onto 20 mL of Ficoll-Hypaque (Pharmacia). After centrifugation at 1500 revolutions per minute for 10 minutes, the buffy coat was washed with PBS, and the cell pellet was frozen in liquid N₂. Total RNA was isolated from

the cell pellet by the standard TRIzol (Gibco BRL) method according to the manufacturer's instructions. Complementary DNA was generated from 1 μ g total RNA via randomly primed reverse transcriptase PCR.

Quantitative PCR for Cadherin-6

The quantitative PCR assay was performed using $\frac{1}{50}$ of the volume of cDNA generated by 1 μ g total RNA in an ABI PRISM 7700 real-time thermal cycler. Upstream and downstream PCR primers and the TagMan probe for cadherin-6 were 5'-GCA CTC ACT ACG CAC A-3', 5'-ACC CTT TGG TGA TCA G-3', and 5'-TGC CAT CAG CAT GAG AAC TTA CCG CTA CTT-3', respectively. TagMan Ribosomal RNA control reagent (Perkin Elmer) was used according to the manufacturer's instructions as an internal control for correction of the mRNA quantity of each sample. A standard curve for the assay was created by quantitative measurement of cadherin-6 mRNA expression in serial dilutions of SKRC-33 total RNA. Quantitative analyses were performed twice for each sample to confirm the reproducibility of the assay.

Evaluation of Cadherin-6 Expression and Correlation between Metastatic Status and Quantity of Cadherin-6 mRNA

The cutoff value of cadherin-6 mRNA expression for this assay was determined by calculating the average value $\pm 2 \times$ the standard deviation (SD) in the healthy control group. The quantity of cadherin-6 mRNA from each blood sample was estimated by comparison with the standard curve of cadherin-6 expression using the SKRC-33 cell line. Comparative analyses of the associations between cadherin-6 levels and the presence and site of metastatic disease were performed. In newly diagnosed patients who had localized or locally advanced RCC, the correlation between the detection of cadherin-6 mRNA and metastases following nephrectomy also was analyzed. In those patients, a plot of recurrence-free survival with metastasis also was constructed using the Kaplan-Meier method, and differences between the two groups were estimated with the log-rank test.

RESULTS

Cutoff Value and Cadherin-6 mRNA Levels in the Samples

Compared with the cutoff values in 25 healthy volunteers, cadherin-6 mRNA levels in 5 additional healthy volunteers were observed at the lower cutoff value. In all patients with malignancies other than RCC, cadherin-6 mRNA levels in peripheral blood samples were within the lower cutoff range (Fig. 1).

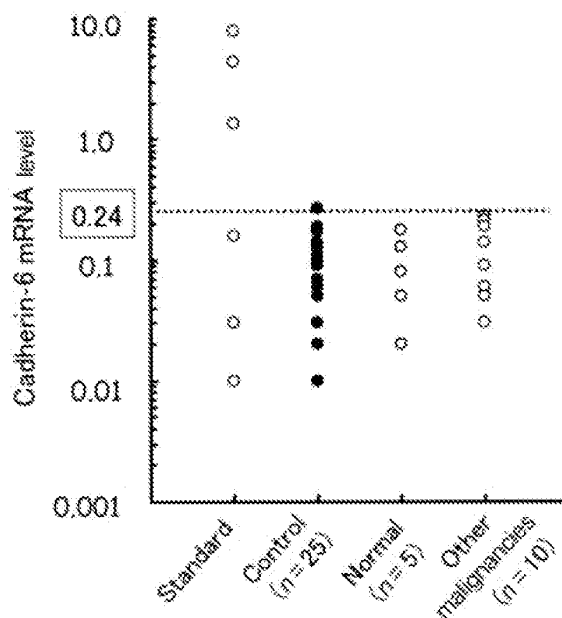


FIGURE 1. Illustration of the cutoff value for cadherin-6 mRNA levels and the validation of this cutoff level using peripheral blood samples from a healthy control group and from a group of patients with urologic malignancies other than renal cell carcinoma. The cutoff value for the assay was 0.24 (the relative score of cadherin-6 mRNA in SKRC-33 cells) and was determined by calculating the mean plus 2 standard deviations in the healthy control group ($n = 25$). Additional samples from the control group and from the group of patients with other malignancies showed the lower level of cutoff value in cadherin-6 mRNA. The y-axis shows the relative score compared with the cadherin-6 mRNA level in the SKRC-33 cell line, and circles in the Standard column indicate serially diluted cadherin-6 mRNA levels in SKRC-33 cells. Y-axis: relative ratio compared to expression level of cadherin-6 in SKRC-33 cells.

Patient Profiles and Metastatic Status during Follow-Up

Of 55 patients with newly diagnosed RCC, 43 patients had no metastatic lesions, and 12 patients had metastases in lung, bone, liver, or other organs. Among the 43 patients who were without metastases, 7 had recurrences with metastatic disease after nephrectomy. Conversely, 11 patients who had recurrences after nephrectomy also had metastases in lung, bone, liver, brain, or other organs.

Quantitative PCR for Cadherin-6 According to the Presence of Metastases

Among the patients who had metastatic disease (including patients with newly diagnosed disease and patients with recurrent disease), 16 of 23 patients (69.9%) were positive for cadherin-6 mRNA, whereas

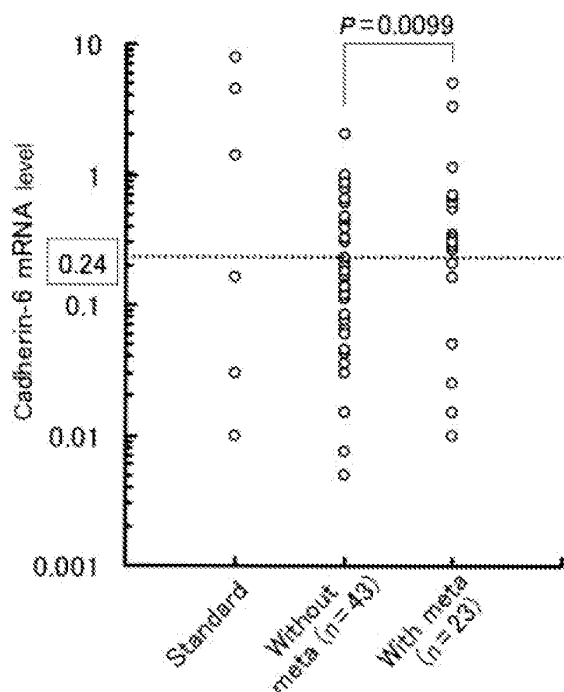


FIGURE 2. Cadherin-6 mRNA levels in peripheral blood from patients with renal cell carcinoma ($n = 66$) according to metastasis (meta) status show that the rate of positive cadherin-6 results among patients who had metastatic disease was statistically greater than the rate among patients without metastatic disease ($P = 0.0099$). Y-axis: relative ratio compared to expression level of cadherin-6 in SKRC-33 cells.

positive expression of cadherin-6 was detected in 15 of 43 patients (34.9%) who were without metastases (newly diagnosed patients). The difference between positive cadherin-6 mRNA rates with and without metastases was statistically significant (chi-square statistic, 7.24; $P = 0.0099$) (Table 1, Fig. 2).

Quantitative PCR for Cadherin-6 According to the Site of Metastases

Detection rates in patients who had metastases after undergoing nephrectomy were 1 in 4 among patients with pulmonary metastases alone and 6 in 7 among patients with metastases to other organs (chi-square statistic, 4.06; $P = 0.044$). Conversely, among the 12 new patients who had metastatic disease, positive cadherin-6 mRNA status before nephrectomy was noted in 9 (Fig. 3). In new patients without metastatic disease, although only 1 of 10 patients with T1a tumors had a positive result, the positive rate was not associated with either T classification or tumor grade.

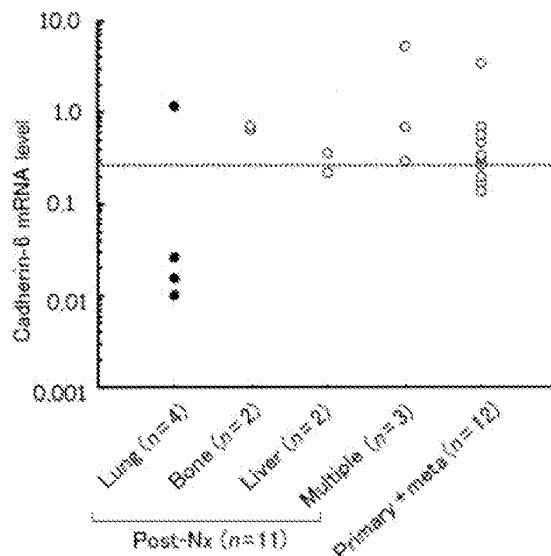


FIGURE 3. Cadherin-6 mRNA levels in patients with metastatic renal cell carcinoma according to the site of metastasis show that a lower positivity rate was observed in patients who had pulmonary metastases (meta) alone. Nx: nephrectomy. Y-axis: relative ratio compared to expression level of cadherin-6 in SKRC-33 cells.

Correlation between the Quantitative Assay for Cadherin-6 and Later Metastases

Among 43 patients with newly diagnosed RCC, a higher rate of recurrence with metastases was observed in patients who had detectable cadherin-6 mRNA levels in peripheral blood compared with patients who had negative cadherin-6 results (5 of 15 patients [33.3%] vs. 2 of 28 patients [7.1%], respectively; $P = 0.0398$) (Fig. 4). The correlation between the detection of cadherin-6 mRNA and later disease recurrence in 26 patients who had both pre-nephrectomy and post-nephrectomy blood samples available is shown in Table 2. Of the six patients who continuously had positive results for cadherin-6, three had metastases. At the time of recurrence, cadherin-6 mRNA remained detectable in five of the seven patients who had positive cadherin-6 status before nephrectomy; in contrast, of the two patients who had negative cadherin-6 status before nephrectomy, only one remained negative for cadherin-6 after this procedure. Recurrence-free survival after nephrectomy in the RCC patient who was positive for cadherin-6 mRNA seemed to be poorer compared with the survival of patients who were negative for cadherin-6. However, the difference between the two groups was not statistically significant ($P = 0.062$; log-rank test) (Fig. 5).

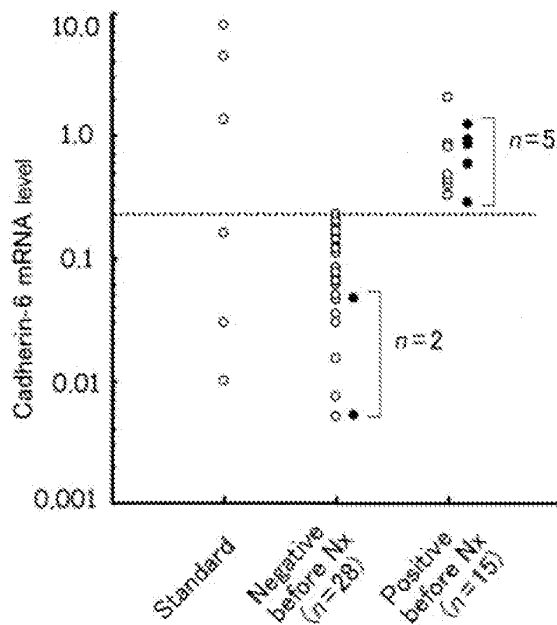


FIGURE 4. Recurrence (black dots) was subsequently documented in 5 of 15 patients who were positive for cadherin-6 mRNA before nephrectomy (Nx), compared with 2 of 28 patients who were negative for cadherin-6. Y-axis: relative ratio compared to expression level of cadherin-6 in SKRC-33 cells.

TABLE 2
Correlation between Detection of Cadherin-6 and Subsequent Metastases

Detection of cadherin-6		Subsequent status (no. of patients)	
Pre-Nx	Post-Nx	Metastasis	No metastasis
Negative	Negative	2	13
Positive	Negative	1	4
Negative	Positive	0	1
Positive	Positive	3	2
Total	Negative or positive	6	20

Nx: nephrectomy.

DISCUSSION

A definitive tumor marker that is expressed in RCC has not been identified to date. Recent studies have demonstrated that RCC cells can be detected in the circulating blood, i.e., from renal and peripheral veins, by means of molecular biologic techniques. McKiernan et al. reported that MN/CA9, which is a relatively specific membrane oncoprotein, is a useful tool for detecting clear cell RCC cells in the peripheral blood independent of tumor stage using reverse transcriptase-PCR (RT-PCR) technology.¹¹ Uemura et al.¹⁰ showed that

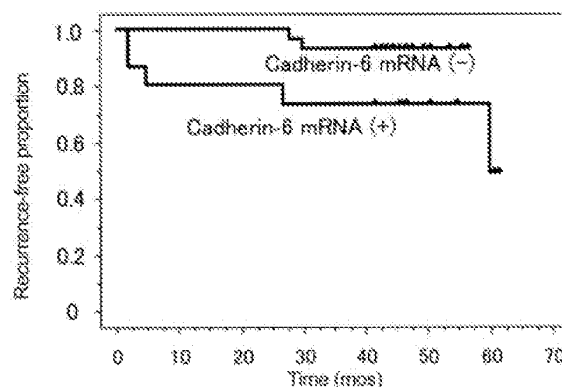


FIGURE 5. Recurrence-free survival curves in patients with renal cell carcinoma after nephrectomy show that the patients who were positive for cadherin-6 mRNA (+; $n = 15$) measured in their peripheral blood seemed to have a higher rate of recurrence compared with patients who were negative for cadherin-6 (-; $n = 28$). However, the difference between the two groups was not significant ($P = 0.062$). Y-axis: Recurrence-free survival rate.

MN/CA9 was expressed in $> 90\%$ of clear cell-type RCCs and was useful for the detection of RCC cells in the peripheral blood.¹³ However, those authors reported that the detection rate of MN/CA9 mRNA in the peripheral blood from healthy volunteers also was relatively high (32.3%), suggesting that positive expression of MN/CA9 is present in normal blood cells or in cells that have been contaminated by the blood sampling procedure.¹⁰ Therefore, this indicator remains controversial among investigators.^{10,11}

Conversely, Ashida et al.¹² demonstrated that the von Hippel-Lindau (*VHL*) gene mutation, which is specific for clear cell-type RCC, is useful for the detection of tumor cells. *VHL* gene mutations were detected in peripheral blood before surgery in 11.8% of patients with sporadic clear cell RCC. This method is considered specific for clear cell RCC, but it is necessary to identify the presence and type of mutation of the *VHL* gene prior to the analysis of blood samples. It is worth noting that the detection rate of RCC cells by *VHL* mutation was remarkably high (77.8%) in renal venous blood during nephrectomy and in peripheral blood in the 24 hours after nephrectomy, indicating that RCC cells may be spread into the circulation system by surgical manipulation. In addition, de la Taille et al.¹⁴ showed that a combined, blood-based RT-PCR analysis of MN/CA9 and prostate-specific membrane antigen is a useful clinical tool for the detection of vascular invasion of RCC, indicating that a PCR-based blood assay using two or three molecules associated with RCC may help to identify micrometastases.

Thus, as shown in our current and previous work,

RCC cells frequently appear in the circulating blood system before and during nephrectomy, independent of the presence of apparent metastatic disease. According to the previous literature, in patients with localized RCC, it is emphasized that the positive rate of RCC cells is between 32% and 46%^{11,14} in peripheral blood but > 70%¹² in renal venous blood during nephrectomy. Because the dissemination of tumor cells into blood circulation is essential in the early stages of metastasis, patients who have localized RCC with positive levels of cadherin-6 mRNA are considered a group that is at high risk for later metastases. Conversely, it also has been speculated that certain molecules in the metastatic cascade, e.g., those of invasion, proliferation, and angiogenesis, are indispensable in the establishment of metastases.

Several factors influence the probability of detection. Tumor volume may be associated with the positive rate of this assay. In the group with metastases, the detection rate was higher in patients who had tumor-bearing kidney compared the rate in patients after nephrectomy. Furthermore, a low detection rate (1 of 10 patients) was observed in patients who had localized T1a tumors that measured < 4.0 cm in greatest dimension. Even in patients with metastatic disease, no definitive reason was identified for the low detection rate in those who had only pulmonary metastases. Our previous study showed that a concentration > 10 cells/mL was required for detection by nested PCR,⁵ indicating that at least 1 RCC cell in 10⁵ mononuclear cells was required for detection using a PCR system, because the mononuclear cell concentration typically ranged between 4000 and 9000 cells/ μ L. Therefore, it is possible that the numbers of circulating tumor cells were not great enough to detect using the PCR system. Alternatively, the level of cadherin-6 mRNA expression of circulating tumor cells may have been too low for detection. The reason for this may be that normal lung epithelial cells express cadherin-6, and RCC cells with cadherin-6 may attach to pulmonary tissue by means of homophilic cadherin adhesion, suggesting that RCC cells that pass through the pulmonary circulation may lack mRNA expression of cadherin-6.

The current quantitative detection system may possess prognostic value, as positive cadherin-6 mRNA status in the blood appeared to be correlated with later recurrence. However, some exceptions were observed in the correlation between the assay results and the occurrence of metastases. This finding may

indicate that the risk of metastasis is associated not only with the presence of tumor cells in the blood circulation but also with the metastatic potential of other tumor cells, e.g., angiogenesis, proliferation, and invasion, as discussed above. Thus, further investigation with longer follow-up will be needed to confirm the predictive value of the quantitative assay for cadherin-6 mRNA.

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Ovarian Carcinoma Subtypes Are Different Diseases: Implications for Biomarker Studies

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Abbreviations: BCCA, British Columbia Cancer Agency; CI, confidence interval; DSS, disease-specific survival; RR, risk ratio; TMA, tissue microarray

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ABSTRACT

Background

Although it has long been appreciated that ovarian carcinoma subtypes (serous, clear cell, endometrioid, and mucinous) are associated with different natural histories, most ovarian carcinoma biomarker studies and current treatment protocols for women with this disease are not subtype specific. With the emergence of high-throughput molecular techniques, distinct pathogenetic pathways have been identified in these subtypes. We examined variation in biomarker expression rates between subtypes, and how this influences correlations between biomarker expression and stage at diagnosis or prognosis.

Methods and Findings

In this retrospective study we assessed the protein expression of 21 candidate tissue-based biomarkers (CA125, CRABP-II, EpCam, ER, F-Spondin, HE4, IGF2, K-Cadherin, Ki-67, KISS1, Matriptase, Mesothelin, MIF, MMP7, p21, p53, PAX8, PR, SLPI, TROP2, WT1) in a population-based cohort of 500 ovarian carcinomas that was collected over the period from 1984 to 2000. The expression of 20 of the 21 biomarkers differs significantly between subtypes, but does not vary across stage within each subtype. Survival analyses show that nine of the 21 biomarkers are prognostic indicators in the entire cohort but when analyzed by subtype only three remain prognostic indicators in the high-grade serous and none in the clear cell subtype. For example, tumor proliferation, as assessed by Ki-67 staining, varies markedly between different subtypes and is an unfavourable prognostic marker in the entire cohort (risk ratio [RR] 1.7, 95% confidence interval [CI] 1.2%–2.4%) but is not of prognostic significance within any subtype. Prognostic associations can even show an inverse correlation within the entire cohort, when compared to a specific subtype. For example, WT1 is more frequently expressed in high-grade serous carcinomas, an aggressive subtype, and is an unfavourable prognostic marker within the entire cohort of ovarian carcinomas (RR 1.7, 95% CI 1.2%–2.3%), but is a favourable prognostic marker within the high-grade serous subtype (RR 0.5, 95% CI 0.3%–0.8%).

Conclusions

The association of biomarker expression with survival varies substantially between subtypes, and can easily be overlooked in whole cohort analyses. To avoid this effect, each subtype within a cohort should be analyzed discretely. Ovarian carcinoma subtypes are different diseases, and these differences should be reflected in clinical research study design and ultimately in the management of ovarian carcinoma.

The Editors' Summary of this article follows the references.

Table 1. Study Population

Clinical Variable	Numerical Display	All	High-Grade Serous	Clear Cell	Endometrioid	Mucinous	Low-Grade Serous
Number of cases	n	500	200	132	129	31	12
Proportion	%	100	40.0	26.4	25.0	6.2	2.4
Age in years	Mean $\pm$ SE	58.1 $\pm$ 0.6	60.9 $\pm$ 0.8	56.2 $\pm$ 1.1	56.0 $\pm$ 1.2	55.4 $\pm$ 2.4	60.2 $\pm$ 4.1
Follow-up time in years	Mean $\pm$ SE	5.9 $\pm$ 0.2	5.4 $\pm$ 0.2	6.3 $\pm$ 0.4	6.4 $\pm$ 0.3	5.4 $\pm$ 0.7	5.8 $\pm$ 1.1
Death	n (%)	233 (46.6)	124 (62.0)	52 (39.4)	39 (31.2)	11 (35.5)	7 (58.3)
Death of disease	n (%)	164 (32.8)	92 (46.0)	40 (30.3)	19 (15.2)	8 (25.8)	5 (41.7)
10 YSR DSS	% $\pm$ SE	57.8 $\pm$ 2.9	38.9 $\pm$ 4.7	63.7 $\pm$ 5.2	83.9 $\pm$ 4.2	72.0 $\pm$ 10.0	48.0 $\pm$ 19.1
Stage I	n (%)	205 (41.0)	49 (24.5)	68 (51.5)	69 (53.2)	18 (58.1)	1 (8.3)
Stage II	n (%)	211 (42.2)	86 (43.0)	56 (42.4)	50 (38.0)	12 (38.7)	7 (58.3)
Stage III	n (%)	84 (16.8)	65 (32.5)	8 (6.1)	6 (4.8)	1 (3.2)	3 (33.3)
Grade 1	n (%)	105 (21.0)	0	0	82 (65.6)	11 (35.5)	12 (100)
Grade 2	n (%)	109 (21.8)	56 (28.0)	0	35 (28.0)	18 (58.1)	0
Grade 3	n (%)	286 (57.2)	144 (72.0)	132 (100)	8 (6.4)	2 (6.5)	0

YSR DSS, year disease-specific survival rate; SE, standard error of the mean.
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Introduction

Ovarian carcinoma is a heterogeneous disease. On the basis of histopathological examination, pathologists classify ovarian carcinoma into serous, clear cell, endometrioid, and mucinous subtypes. Each of these subtypes is associated with different genetic risk factors and molecular events during oncogenesis [1,2], and characterized by distinct mRNA expression profiles [3,4]. These subtypes differ dramatically in frequency, when early stage carcinomas (where the majority are nonserous carcinomas [5]) and advanced stage carcinomas (which are predominantly of serous subtype [6]) are compared.

Oncologists have noted that subtypes respond differently to chemotherapy. The dismal response rate of clear cell carcinomas (15%) contrasts sharply with that of high-grade serous (80%), resulting in a lower 5-y survival for clear cell compared with high-grade serous carcinoma in patients with advanced stage tumors (20% versus 30%) [7,8]. Therefore, the National Cancer Institute (NCI) State of Science meeting recently singled out clear cell carcinoma as a candidate for clinical trials to identify more active therapy than what is currently available [9]. Although these data suggest substantial differences between subtypes, ovarian carcinoma is typically approached as a monolithic entity by researchers and clinicians. This practice impedes progress in understanding the biology or improving the management of the less common ovarian carcinoma subtypes.

We hypothesized that correlations between biomarker expression and stage at diagnosis or prognosis would reflect subtype variation in biomarker expression. To test this hypothesis we correlated protein expression rates of a panel of 21 candidate biomarkers with stage at diagnosis and disease-specific survival (DSS) in a large cohort of ovarian carcinomas and also analyzed these associations within ovarian carcinoma subtypes.

Methods

Study Population

The Cheryl Brown Ovarian Cancer Outcomes Unit is an ovarian cancer registry serving a population of approxi-

mately four million people in British Columbia. For the period 1984–2000, 2,555 patients with ovarian carcinoma were recorded in the registry. From these 834 patients were selected based on the criterion being free of macroscopic apparent residual disease after primary surgery and all histological slides underwent gynecopathological review. Subtypes were assigned according to refined World Health Organization (WHO) criteria [10] as recently described [5]. A further 91 patients diagnosed in stage Ia or Ib, grade 1 were excluded from the study because of excellent prognosis; only 3% of women in this group died of disease during the follow-up period. From the remaining patients 541 tissue blocks were available and used for tissue microarray (TMA) construction. A representative area of each tumor was selected and duplicate 0.6-mm tissue cores were punched to construct a TMA (Beecher Instruments). Review after TMA construction revealed that 23 cases were not adequately sampled. Of these 23 cases, 20 mixed carcinomas (>10% of tumor showing a second histological cell type) were excluded because their highest grade component was not sampled on the TMA; 18 cases were either of rare histological types (including seven undifferentiated, six transitional, and one squamous carcinoma) or could not be specified (five cases). This approach resulted in a study population of exactly 500 cases belonging to one of the four major cell types (serous, endometrioid, clear cell, and mucinous) (Table 1). The serous subtype was further subdivided into low- and high-grade [11]. Two cases of endometrioid carcinomas containing minor mucinous or low-grade serous components (>10%) are included in the study.

Adjuvant Therapy and Follow-up

All patients received standardized treatment according to the provincial treatment guidelines of the British Columbia Cancer Agency (BCCA) [12,13]; however, 3% of patients refused the advised adjuvant chemotherapy and were excluded from survival analysis. For 3% adjuvant therapy was not advised, hence 94% received platinum-based chemotherapy (with or without abdomino-pelvic radiotherapy) adjuvant treatments. Outcomes were tracked via the Cheryl Brown Ovarian Cancer Outcomes Unit at the BCCA and were available for all patients. Follow-up information was obtained

Table 2. Antibodies

Number	Biomarker	Supplier	Clone	Dilution	Full Name/Description
1	CA125	Cellmarque	OC125	1:100	Cancer antigen 125, cell surface glycoprotein
2	CRABP-II	Santa Cruz	Polyclonal	1:25	Cellular retinoic acid-binding protein II, transcriptional regulator of lipid metabolism
3	EpCam	R&D Systems	158206	1:25	Epithelial cell adhesion molecule, cell-cell adhesion
4	ER	Labvision	5P1	1:200	Estrogen receptor
5	F-Spondin	US Biological	Polyclonal	1:50	Neuronal development
6	HE4	Signet	Polyclonal	1:25	Human epididymis protein 4 is a member of 4-disulfide core protein with unknown function
7	IGF2	Abcam	Polyclonal	1:100	Insulin-like growth factor 2
8	K-Cadherin	Abcam	2B6	1:50	Cell-cell adhesion protein
9	Ki-67	Labvision	SP6	1:200	MKI, proliferation-associated antigen detected by Ki67
10	KISS1	Santa Cruz	Polyclonal	1:400	Kisspeptins, ligands of G-protein coupled receptor 54
11	Matrilysin	Bethyl	Polyclonal	1:25	Type II transmembrane trypsin-like serine protease, degradation of extracellular matrix
12	Mesothelin	Novocastra	5B2	1:50	Cell surface glycoprotein
13	MIF	R&D Systems	Polyclonal	1:2500	Macrophage inhibitory factor, modulator of chronic inflammation
14	MMP7	Chemicon	141-782	1:200	Matrix metalloproteinase 7, degradation of extracellular matrix
15	p21	Labvision	DC5-60.2	1:40	Cyclin-dependent kinase inhibitor 1A (Cip1)
16	p53	DAKO	DO-7	1:400	Tumor protein p53
17	PAX8	Donation*	Polyclonal	1:500	Thyroid specific transcription factor, Pax8/PPARGgamma fusion gene in 50% of follicular thyroid carcinomas
18	PR	Labvision	SP2	1:400	Progesterone receptor
19	SLPI	Hycult	31	1:100	Secretory leukocyte protease inhibitor
20	TROP2	R&D Systems	Polyclonal	1:25	Tumor-associated calcium signal transducer 2
21	WT1	DAKO	6F-H2	1:100	Wilms tumor suppressor 1, zinc finger transcription factor

*The α -mPax8-bIII antibody was kindly provided by Roberto Di Lauro, Stazione Zoologica, Naples, Italy.
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through the electronic patient record of the BCCA or the patient's paper chart. Examples of documentation used to ascertain vital status include BCCA progress notes, death certificates, and correspondence indicating status from other care providers. Ovarian carcinoma specific death was defined where ovarian cancer was the primary or underlying cause of death. Death from concurrent disease (i.e., second malignancy) was coded as "died of other cause." Death resulting from toxicities relating to treatments for ovarian carcinoma was coded as "died of toxicities." Abstracted data were reviewed by an experienced medical oncologist (K.S.). Median follow-up time was 5.1 y. Approval for the study was obtained from the Research Ethics Board of the University of British Columbia.

Marker Selection and Immunohistochemistry

The goal of our marker selection was to use proteins that are consistently expressed in ovarian carcinomas and have been reported as prognosticators (p53, p21, Ki-67, PR, WT1) [14–19] or being developed as early detection markers in ovarian carcinomas [20]. This approach biased our results towards selection of markers mostly derived from and expressed in high-grade serous subtype. Serial 4- μ m sections were cut for immunohistochemical (IHC) analysis and run through an automated protocol including heat antigen retrieval (Ventana System). The antibodies and suppliers are listed in Table 2. Specificity was determined by using appropriate positive controls, with omission of primary antibody as a negative control.

Evaluation of Immunohistochemistry

One or more pathologists (MK, DNI, or AR) scored these biomarkers after scanning with a BLISS scanner (Bacus

Laboratories/Olympus America). Except KISS1 [21] and p53 [22] where recently published cut-off points were used, all markers were dichotomized into negative and positive cases (cut-off values for positive versus negative for all markers except Ki-67 are shown in Table S1). Ki-67 was assessed as a continuous variable as a percentage of positive tumor cells using automated image analysis software [23]. Prior to analysis a pathologist (MK) manually selected regions of interest so as to avoid noncancerous cellular areas. The median was used to dichotomize into low- and high-expressing groups for Ki-67.

Statistical Analysis

Contingency analysis and Pearson's χ^2 statistic were used to test the change in the distribution of biomarker expression across stage and subtypes. The Kruskal-Wallis test was used to determine if Ki-67 was differentially expressed across stage and subtypes. Univariable DSS was illustrated by the generation of Kaplan-Meier curves and subgroup differences tested with a univariable Cox model. Multivariable DSS was tested using the Cox proportional hazards model. The Cox proportional hazards model was used to determine risk ratios (RRs) and p -values for all univariable and multivariable DSS analyses. Additionally, to assess significance in the presence of some small subgroups, permutation tests were performed and permutation p -values reported. Under the null hypothesis of no association of biomarker status with survival (for survival analyses) or stage/histology (for contingency table analyses), the biomarker outcomes are exchangeable across cases. For the survival analyses, permutations of biomarker outcomes were performed within stage/subtype subgroups, to preserve the observed distribution of biomarker frequencies within

subgroups. Permutation was performed by exchanging each case's entire biomarker panel at random without replacement among cases, to preserve correlation structure within case. A total of 10,000 permutation replications were performed. *p*-Values were obtained by finding the number of permutation sample estimates (Cox model parameter estimate for survival analyses, Pearson χ^2 statistic for contingency table analyses) as extreme or more extreme than the observed value. $p < 0.05$ was considered statistically significant. Hence, any prognostic correlations for a single biomarker have to be interpreted with caution. Statistical analyses were performed using SPSS software (version 15.0; SPSS) and R (version 2.5.1; R Foundation for Statistical Computing).

Results

Biomarker Expression Profile Reflects Subtype

This cohort of 500 ovarian carcinomas was mainly selected based on the criterion of not having apparent residual tumor after primary surgery. Since successful surgery is typically achieved in lower stage, this case selection strategy can be anticipated to include more cases of tumors of histological subtypes that are commonly diagnosed at low stage, such as clear cell carcinoma (26.4%), endometrioid (25.0%), and mucinous (6.2%) carcinomas, although serous carcinomas were still the most common subtype (40.0% high-grade and 2.4% low-grade) in this cohort (Table 1).

Interpretable results of immunostains for the 21 candidate biomarkers (Figure 1) ranged from 363 to 493 (median 488, Table S2). The larger numbers of missing data for three biomarkers were caused by exhaustion of tumor material in the core. All immunostains with annotated clinical information are available online at <http://www.gpecimage.ubc.ca> (username: BCCA-VGH; password: OVCARE). The rate of positive cases for each biomarker ranged from 9% (KISS1) to 83% (EpCam) (detailed expression rates are listed in Table S2). Comparing biomarker expression in the entire cohort for tumors diagnosed at different stages revealed that ten biomarkers (CRABP-II, ER, F-Spondin, K-Cadherin, Ki-67, Matriptase, Mesothelin, p21, p53, and WT1) had significantly different expression levels between stages, suggesting differences between "early" and "late" stage disease (Figure 2, Table S2). However, comparing biomarker expression within one subtype across FIGO stages, no biomarker remained significantly differently expressed by stage (results for high-grade serous subtype are shown in Figure 3). This result was true for all four major subtypes (unpublished data for endometrioid, clear cell, and mucinous). In contrast, 20 of 21 biomarkers were significantly differentially expressed between the subtypes (Figure 4). Only, EpCam ($p = 0.23$) showed a consistent expression frequency across all subtypes. Additionally, *p*-values for biomarker expression rates in the entire cohort across subtypes were generally smaller than across stages (Table S2), indicating a stronger association with subtype than stage.

High-grade serous carcinoma showed positive staining in >75% of cases for WT1, Mesothelin, ER, and CA125 (Table S2). The biomarker expression pattern of low-grade serous carcinomas was similar to that of their high-grade counterparts. Three markers (PR, p53, K-Cadherin) showed a trend towards differential expression in low-grade versus high-grade serous subtypes. Only the median Ki-67 labelling index

differed significantly between those groups, with median Ki-67 labelling index of 2.5% (95% confidence interval [CI] 0.5%–20.4%) in low-grade serous versus 22.4% (95% CI 3.6%–69.9%) in high-grade serous subtype (Figure 5). Endometrioid carcinomas coexpress high rates of hormone receptors ER and PR as well as CA125. Endometrioid and clear cell subtypes infrequently (<10%) expressed WT1 and p53. The median Ki-67 labelling index for endometrioid and clear cell carcinomas was similar (endometrioid 8.2%, 95% CI 0.8%–49.0%; clear cell 7.6%, 95% CI 0.5%–45.0%). Immunophenotypic characteristics of clear cell carcinomas included low levels of hormone receptors ER (10%) and PR (3%). The mucinous subtype displayed an intermediate proliferative capacity compared with the other subtypes (median Ki-67 labelling index 12.9%, 95% CI 2.1%–60.9%) and frequent expression of Matriptase (86%). Many of the markers expressed in other subtypes were either infrequently (<10%) expressed (p53, ER, PAX8, SLPI, K-Cadherin, and CA125), or completely absent (CRABP2, WT-1, and Mesothelin). Of note, EpCam was highly expressed across all subtypes included in this study.

Survival Analyses Can Be Confounded by Subtype Differences

To assess the biological importance of a biomarker, its expression is usually correlated with outcome. Survival analysis was restricted to the three major subtypes (high-grade serous, clear cell, and endometrioid) because of insufficient numbers of cases of mucinous or low-grade serous subtypes. The primary endpoint was defined as DSS and the rates after 10 y are shown for subtypes in Table 1. A multivariable Cox regression model including age, stage, and histological subtype showed significant differences across stage ($p < 0.0001$) and subtype ($p = 0.015$). Survival by stage showed little difference between stages I and II, with stage III showing poorer DSS (RR 3.0, 95% CI 1.87%–4.66% relative to stage I). Survival by subtype showed poorer DSS for clear cell (RR 2.31, 95% CI 1.29%–4.15%) and high-grade serous (RR 2.74, 95% CI 1.56%–4.81%) relative to endometrioid subtype. Age was not predictive in the model ($p = 0.211$) (Table S3).

Univariable Cox regression analysis for each biomarker was applied on the entire cohort as well as within the three largest subtypes (Figure S1, Table 3). RRs and *p*-values are presented in Table 3. Nine of 21 biomarkers show prognostic significance in the entire cohort. Of the nine biomarkers showing a significant association with DSS in the entire cohort, three remain prognostic indicators in the high-grade serous and one in the endometrioid subtype. As an extreme example, WT1 is an unfavourable prognostic biomarker in the entire cohort ($p = 0.0017$, Figure 6A) but is a favourable prognostic biomarker for high-grade serous carcinomas ($p = 0.0086$, Figure 6B). As WT1 is expressed in 80% of high-grade serous carcinomas but rarely in other subtypes, this negative prognostic significance in the entire cohort reflects subtype differences in expression, with WT1 most commonly expressed in the aggressive high-grade serous subtype. Four other biomarkers (KISS1, K-Cadherin, Mesothelin, Ki-67) that were significant in the entire cohort did not show significance in any subtype.

Ki-67 serves as an additional example, which is prognostic in the whole cohort but not when corrected for subtype. The median for Ki-67 labelling index in the entire cohort was

Ovarian Carcinoma Subtypes Are Different

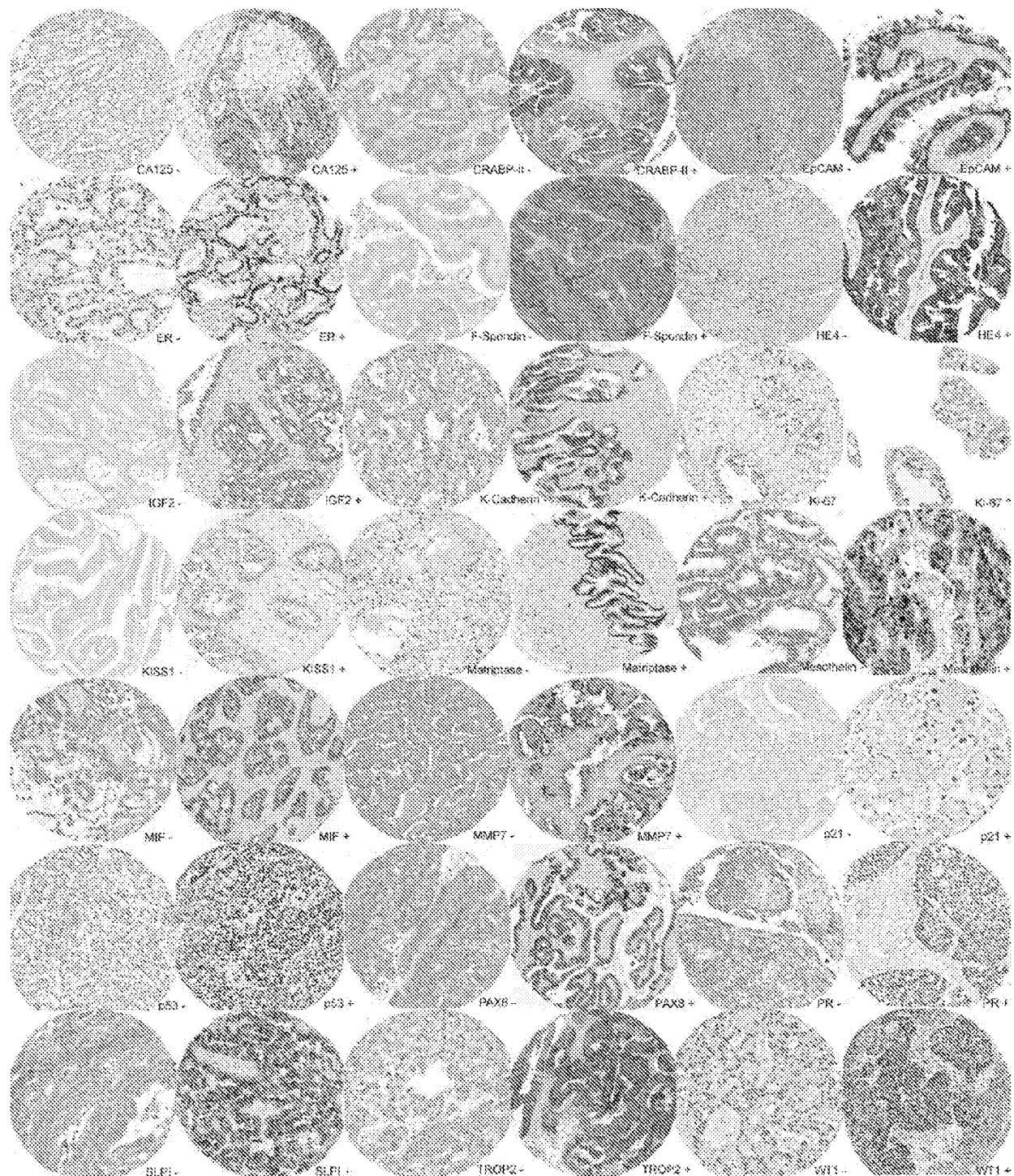


Figure 1. Representative Immunostains
Paired positive and negative examples for each biomarker.
doi:10.1371/journal.pmed.0050232.g001

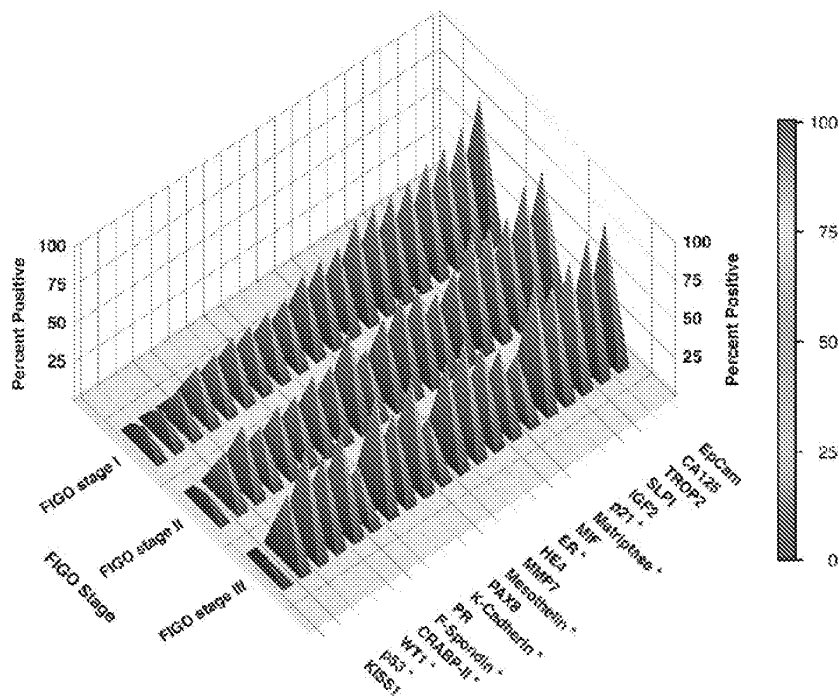


Figure 2. Biomarker Expression Rates in the Entire Cohort by Stage
 *Significant differences between categories (Fisher's exact test).
 doi:10.1371/journal.pmed.0050232.g002

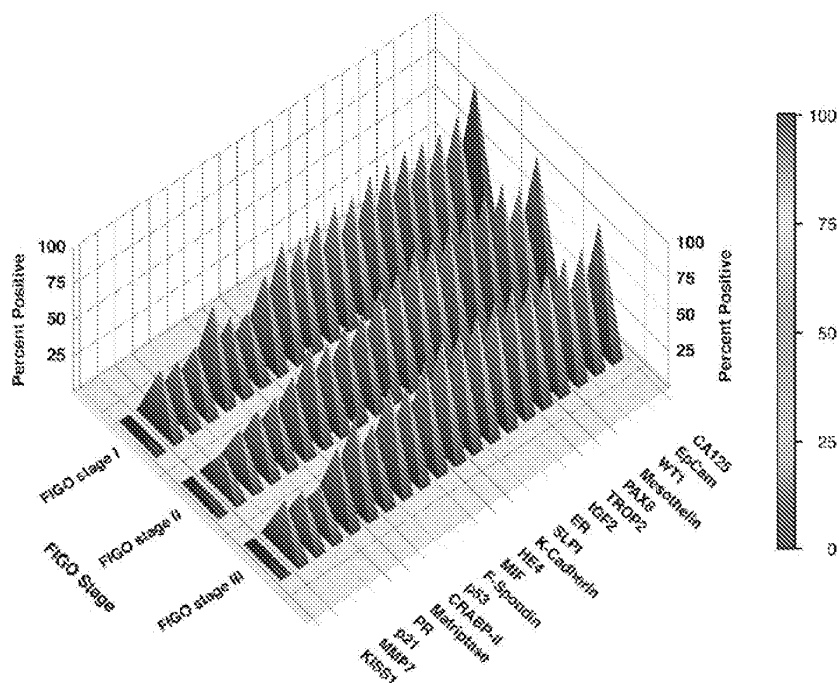


Figure 3. Biomarker Expression Rates in High-Grade Serous Subtype by Stage
 doi:10.1371/journal.pmed.0050232.g003

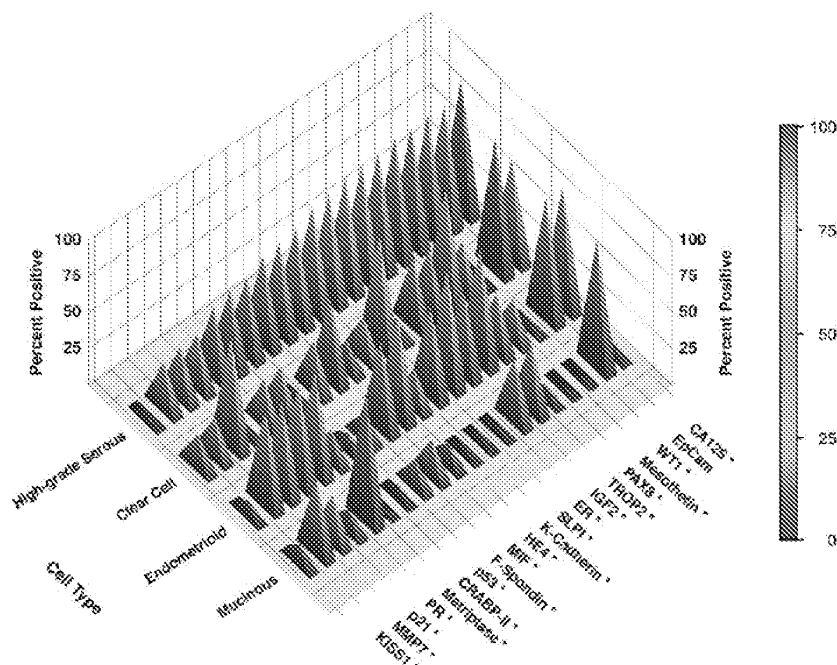


Figure 4. Biomarker Expression Rates in the Entire Cohort by Subtype

*Significant differences between categories (Fisher's exact test). Note that the order in which biomarkers are presented is based on percentage of positivity and that therefore the order is different in Figures 2–4.
doi:10.1371/journal.pmed.0050232.g004

13.0% and using this as a cut-off for high versus low Ki-67 labelling index effectively separates high-grade serous carcinomas from low-grade serous, endometrioid, and clear cell carcinomas (Figure 5). Mucinous carcinomas showed an intermediate Ki-67 labelling index. Associated with high-grade serous subtype, it is not surprising that Ki-67 has prognostic relevance in the whole cohort ($p = 0.0062$). When using the subtype specific median for separate analysis of each subtype however, Ki-67 labelling index was not of

prognostic significance in any of the subtypes but Ki-67 labelling index was different between subtypes.

Discussion

Ovarian carcinomas subtypes are associated with distinct genetic risk factors, underlying molecular events during oncogenesis, stage at diagnosis, and responses to chemotherapy. With slight modification of the WHO criteria for histopathological assignment for subtype we have recently shown that classification of ovarian carcinomas into five subtypes (high-grade serous, low-grade serous, clear cell, endometrioid, and mucinous) is reproducible and is supported by biomarker expression data [5].

By demonstrating that biomarker correlations with stage or prognosis can be explained by variations in expression rates between subtypes, our study offers persuasive evidence supporting the view that ovarian carcinoma subtypes are different diseases. Biomarker expression is stable across stage within a given subtype. Furthermore, differences in the expression profile between subtypes confound survival analysis for biomarkers, when multiple ovarian carcinoma subtypes are considered together. Collectively, these data have implications for ovarian carcinoma research and treatment.

Cancer treatment in general is beginning to move towards therapies tailored for specific cancer subtypes (e.g., breast carcinoma and lymphoma [24,25]), and this subtype specific approach to treatment has implications for the design of clinical trials for women with ovarian carcinomas. It has been recognized for some time that certain ovarian carcinoma subtypes are less sensitive to platinum-based chemotherapy

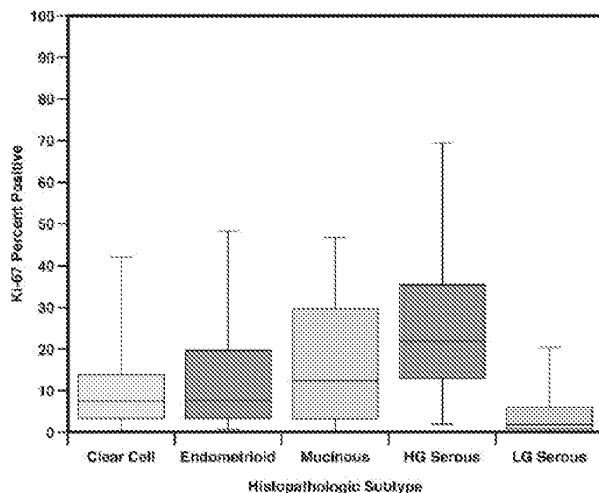


Figure 5. Distribution of Ki-67 Labelling Index across Subtypes

doi:10.1371/journal.pmed.0050232.g005

Table 3. Univariable COX Regression for Disease-Specific Survival

Number	Biomarker	Risk Factor	Entire Cohort (n = 500)	High-Grade Serous (n = 200)	Clear Cell (n = 132)	Endometrioid (n = 125)
			RR (95% CI)	RR (95% CI)	RR (95% CI)	RR (95% CI)
1	CA125	Positive	1.23 (0.82–1.85)	0.32	0.51	0.89
2	CRABP-II	Positive	1.35 (0.97–1.88)	0.071	0.84	0.63
3	Epcam	Negative	1.38 (0.92–2.09)	0.12	0.082	0.77
4	ER	Negative	1.07 (0.77–1.47)	0.70	0.48	0.83
5	F-Spondin	Positive	1.54 (1.07–2.20)	0.020	0.981	0.81
6	HER4	Negative	1.12 (0.78–1.60)	0.54	0.45	0.94
7	Ki67	Positive	1.22 (0.85–1.74)	0.28	0.98	0.64
8	K-Cadherin	Positive	1.47 (1.07–2.04)	0.018	0.72	0.37
9	Ki-67	High	1.74 (1.25–2.43)	0.00062	0.39	0.75
10	KISS1	Low	3.42 (1.51–7.77)	0.0033	0.0078	0.067
11	Matriptase	Negative	1.63 (1.13–2.36)	0.0089	0.047	0.28
12	Mesothelin	Positive	1.65 (1.19–2.29)	0.0030	0.31	0.86
13	MIF	Positive	1.11 (0.80–1.54)	0.53	0.50	0.30
14	MMP7	Negative	2.44 (1.68–3.56)	<0.0001	0.0006	0.44
15	p21	Negative	1.30 (0.94–1.79)	0.11	0.95	0.77
16	p53	High	1.41 (0.99–2.00)	0.051	0.48	0.44
17	PAX8	Positive	1.38 (0.97–1.94)	0.071	0.94	0.95
18	PR	Negative	1.59 (1.10–2.28)	0.013	0.32	0.77
19	SLP1	Negative	1.02 (0.72–1.42)	0.92	0.76	0.31
20	TRCP2	Negative	1.08 (0.77–1.52)	0.65	0.48	0.59
21	WT1	Positive	1.66 (1.21–2.29)	0.0017	0.25	0.13

*Raw unadjusted asymptotic p-values.

*Permutation test p-values.

doi:10.1371/journal.pmed.1000122.t003

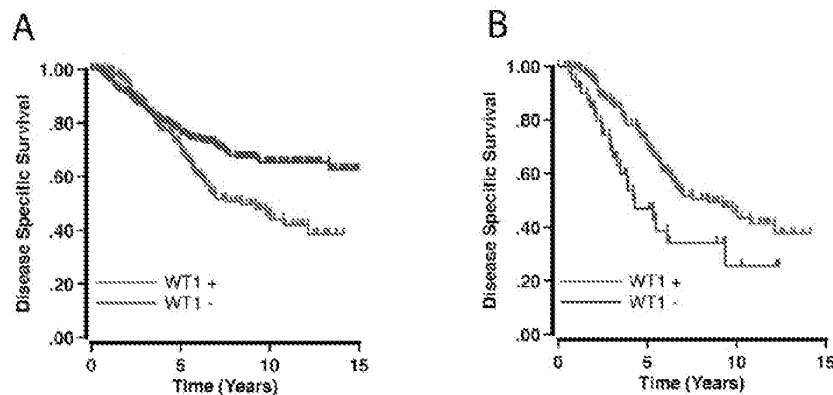


Figure 6. Prognostic Associations of WT1

Kaplan-Meier survival analysis of DSS.

(A) Entire cohort grouped by WT1 positive versus negative cases ($p = 0.0017$, univariable COX regression).

(B) high-grade serous subtype grouped by WT1 positive versus negative cases ($p = 0.0086$, univariable COX regression).

doi:10.1371/journal.pmed.0050232.g006

than the most common high-grade serous carcinomas. The clear cell and mucinous subtypes, in particular, are candidates for clinical trials to identify more active therapy than what is currently used [9]. Given the dramatic differences in biomarker expression between ovarian carcinoma subtypes, our analysis suggests that advancing our understanding of these poorly understood subtypes—including identification of potential therapeutic targets—will only come through studies focusing on these specific subtypes rather than studies of unselected series of patients.

The biomarker expression profile within a given subtype is consistent across stage. Hence, early and advanced stage ovarian carcinomas differ primarily based on subtype, while within a subtype there is no difference between early and advanced stage tumors. This distinction has implications for the research on biomarkers for ovarian carcinoma screening, where the goal is detection of early stage disease, which has a much greater likelihood of cure. If subtypes are neglected, a screening marker identified in advanced stage tumors (i.e., high-grade serous carcinomas), may not be expressed in most nonserous early stage ovarian carcinomas, and vice versa. For example, CA125 is expressed in most high-grade serous carcinoma, but only in 60% of mucinous and clear cell subtypes, a finding that is consistent with previous studies [26]. A related observation is that serum CA125 levels are elevated in 80% of patients with advanced stage epithelial ovarian carcinoma but are increased in only 60% of patients with early stage disease [27,28]. It is likely that a panel of tumor markers will be required to detect all subtypes. As the biomarker expression was consistent between stages within the subtypes, these data support the use of late stage cancers to identify biomarkers for the early detection of cancers of the same subtype.

Biomarker correlation with prognosis can be confounded by subtype differences in biomarker expression. Some biomarkers show prognostic significance independent from subtype, e.g., we confirmed that MMP7 expression is a strong independent prognostic factor for favourable prognosis in the entire cohort, as shown previously [29] as well as for high-grade serous and endometrioid subtypes. This result is the exception rather than the rule as for most of the biomarkers,

the correlation with prognosis in the entire cohort is due to the correlation with the most common subtype (high-grade serous carcinoma), which in turn is associated with a poor prognosis. The biomarkers that were of prognostic significance in subtype analysis were typically only of prognostic value for a single subtype. WT1 is a widely used diagnostic marker for the serous subtype [30] and is an example for how analysis of the entire cohort can give misleading results. In the entire cohort WT1 is an unfavourable prognostic marker but is a favourable prognostic marker for high-grade serous tumors (Figure 6). This latter observation may be because WT1 is a marker for serous differentiation, and less differentiated high-grade serous cancers are both less likely to express WT1 and have a worse prognosis. This inverse association in a subgroup, also known as Simpson's paradox, will not typically be revealed by multivariable analysis [19].

Another example is Ki-67; there are conflicting results on the prognostic value of Ki-67 in ovarian carcinoma [31–36]. After applying a single cut-off point on the entire cohort for identification of Ki-67 high and Ki-67 low cases, high Ki-67 index is associated with an unfavourable prognosis. But differences in Ki-67 indices between subtypes again confound the analysis because nearly all high-grade serous carcinomas have a high Ki-67 index. In analysis by subtype, Ki-67 is not of prognostic significance; the effect seen in the entire cohort reflects an association with the high-grade serous subtype.

Adjustment for multiple comparisons is an important consideration. However, there are several bodies of data under discussion in different sections of this report, with differing numbers of comparisons. For example, assessing the proportion of positive cases across histological subtypes for each biomarker involves the assessment of 21 tests; whereas assessing survival within FIGO and histological subtype groups involves more tests. Since p -value adjustment for multiple testing uses the number of tests under consideration, several collections of adjusted p -values would have to be constructed yielding a complex distraction from the discussion at hand. We note that for the assessment of proportion of positive cases, the Bonferroni-adjusted level of significance would be $0.05/21 = 0.0024$, and several p -values in that analysis are less than this level. We report raw p -values so

that the reader can apply multiple comparison adjustments relevant to the size of comparisons being made in any section of the paper, and in future meta-analyses of subsets of these data. Corrections for multiple comparisons were not used because this issue of prognostic significance is not the central theme of the manuscript. Prognostic significance is used to illustrate the importance of subtype-specific analysis. A limitation of our study is that it is performed retrospectively [37], and 94% of patients received adjuvant platinum-based chemotherapy. Hence, we can not adhere to the strict definition of a prognostic marker as applying only to the natural history of the disease.

We hope these data will end the lumping of ovarian carcinoma subtypes within biomarker studies, as is the current practice [38–41]. This biomarker panel shows that subtypes have distinct expression profiles. One of the reasons why no ovarian carcinoma tissue-based prognostic markers are used clinically, despite a voluminous literature suggesting many candidates, is that prognostic effects have proven difficult to validate. In addition to assay specific challenges, the different frequencies of subtypes within cohorts can vary or, as shown here with WT1, reverse prognostic effects. If ovarian carcinoma cases are not separated by subtype or evaluated using a stratified analysis or a model with complex interaction terms, even a multivariable model can conceal important findings or lead to misleading conclusions (Table S4). The discovery, development, and validation of subtype specific ovarian carcinoma biomarkers will require adequately powered and expertly subtyped cohorts of cases. For the rarer subtypes, the development of such research resources will likely prove difficult outside of large scale collaborative initiatives. In order to facilitate the shift to subtype specific management of ovarian carcinoma, subtypes should be considered as distinct diseases in biomarker studies and clinical trials.

Supporting Information

Figure S1. Kaplan-Meier Survival Analysis of Disease-Specific Survival in the Entire Cohort, Stage Subgroups, Subtypes, and the Stage Subgroups by Subtype

p-Values (Wald) were generated using a multivariable Cox regression model including the biomarker and age. "Marker * CellType Xn *p*"-value assesses differential biomarker prognostic value in the different subtypes (a large *p*-value indicates that biomarker prognosis is similar in the subgroups, a small *p*-value indicates that biomarker prognosis differs in the subgroups); "Marker * FIGO Xn *p*"-value assesses differential biomarker prognostic value in the different subgroups. HC-SC, high-grade serous; EC, endometrioid; CC, clear cell; FIGO=stg12, FIGO stage I and II; FIGO=stg3, FIGO stage III.

Found at doi:10.1371/journal.pmed.0050232.s001 (2 MB PDF).

Table S1. Definition of Positive Staining

Found at doi:10.1371/journal.pmed.0050232.s001 (42 KB DOC).

Table S2. Biomarker Expression Rate Across Stage, Subtype, and Stage within Subtypes

Found at doi:10.1371/journal.pmed.0050232.s002 (133 KB DOC).

Table S3. Multivariable COX Proportional Hazards Including Stage, Subtype, and Age for the Entire Cohort

Found at doi:10.1371/journal.pmed.0050232.s003 (30 KB DOC).

Table S4. Multivariable COX Proportional Hazards Including Stage, Subtype, Age, and WT-1 for the Entire Cohort and High-Grade Serous Carcinomas

Found at doi:10.1371/journal.pmed.0050232.s004 (38 KB DOC).

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Author contributions. DH conceived the study. MK, CBG, and DH designed the study. MK, SEK, NB, SM, EM, CP, SL, NJB, DNI, AR, LMP, DM, JS, KS, and DH were involved in acquisition of data and analysis/interpretation of the data. MK had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of data analysis. SL created companion website. MK and CBG wrote the first draft of the manuscript. MK, SEK, CP, LMP, and DH participated in revising the manuscript. MK, SEK, NB, SM, EM, CP, SL, NJB, DNI, AR, LMP, DM, JS, KS, CBG, and DH approved submission of the final draft.

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Editors' Summary

Background. Every year, about 200,000 women develop ovarian cancer and more than 100,000 die from the disease. Ovarian epithelial cancer (carcinoma) occurs when epithelial cells from the ovary or fallopian tube acquire mutations or equivalent changes that allow them to grow uncontrollably within one of the ovaries (two small organs in the pelvis that produce eggs) and acquire the potential to spread around the body (metastasize). While the cancer is confined to the ovaries, cancer specialists call this stage I disease; 70%–80% of women diagnosed with stage I ovarian cancer survive for at least 5 y. However, only a fifth of ovarian cancers are diagnosed at this stage; in the majority of patients the cancer has spread into the pelvis (stage II disease), into the peritoneal cavity (the space around the gut, stomach, and liver; stage III disease), or metastasized to distant organs such as brain (stage IV disease). This peritoneal spread might be associated with often only vague abdominal pain and mild digestive disturbances. Patients with advanced-stage ovarian carcinoma are treated with a combination of surgery and chemotherapy but, despite recent advances in treatment, only 15% of women diagnosed with stage IV disease survive for 5 y.

Why Was This Study Done? Although it is usually regarded as a single disease, there are actually several distinct subtypes of ovarian carcinoma. These are classified according to their microscopic appearance as high-grade serous, low-grade serous, clear cell, endometrioid, and mucinous ovarian carcinomas. These subtypes develop differently and respond differently to chemotherapy. Yet scientists studying ovarian carcinoma usually regard this cancer as a single entity, and current treatment protocols for the disease are not subtype specific. Might better progress be made toward understanding ovarian carcinoma and toward improving its treatment if each subtype were treated as a separate disease? Why are some tumors confined to the ovary, whereas the majority spread beyond the ovary at time of diagnosis? In this study, the researchers address these questions by asking whether correlations between the expression of “biomarkers” (molecules made by cancer cells that can be used to detect tumors and to monitor treatment effectiveness) and the stage at diagnosis or length of survival can be explained by differential biomarker expression between different subtypes of ovarian carcinoma. They also address the question of whether early stage and late stage ovarian carcinomas are fundamentally different.

What Did the Researchers Do and Find? The researchers measured the expression of 21 candidate protein biomarkers in 500 ovarian carcinoma samples collected in British Columbia, Canada, between 1984 and 2000. For 20 of the biomarkers, the fraction of tumors expressing the

biomarker varied significantly between ovarian carcinoma subtypes. Considering all the tumors together, ten biomarkers had different expression levels in early and late stage tumors. However, when each subtype was considered separately, the expression of none of the biomarkers varied with stage. When the researchers asked whether the expression of any of the biomarkers correlated with survival times, they found that nine biomarkers were unfavorable indicators of outcome when all the tumors were considered together. That is, women whose tumors expressed any of these biomarkers had a higher risk of dying from ovarian cancer than women whose tumors did not express these biomarkers. However, only three biomarkers were unfavorable indicators for high-grade serous carcinomas considered alone and the expression of a biomarker called WT1 in this subtype of ovarian carcinoma is associated with a lower risk of dying. Similarly, expression of the biomarker Ki-67 was an unfavorable prognostic indicator when all the tumors were considered, but was not a prognostic indicator for any individual subtype.

What Do These Findings Mean? These and other findings indicate that biomarker expression is more strongly associated with ovarian carcinoma subtype than with stage. In other words, biomarker expression is constant from early to late stage, but only within a given subtype. Second, the association of biomarker expression with survival varies between subtypes, hence lumping all subtypes together can yield misleading results. Although these findings need confirming in more tumor samples, they support the view that ovarian carcinoma subtypes are different diseases. In practical terms, therefore, these findings suggest that better ways to detect and treat ovarian cancer are more likely to be found if future biomarker studies and clinical research studies investigate each subtype of ovarian carcinoma separately rather than grouping them all together.

Additional Information. Please access these Web sites via the online version of this summary at <http://dx.doi.org/10.1371/journal.pmed.0050232>.

- The US National Cancer Institute provides a brief description of what cancer is and how it develops and information on all aspects of ovarian cancer for patients and professionals. It also provides a fact sheet on tumor markers (in English and Spanish).
- The UK charity Cancerbackup provides general information about cancer and more specific information about ovarian cancer, including tumor staging.

ORIGINAL ARTICLE

Cadherin-6 promotes EMT and cancer metastasis by restraining autophagy

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The transdifferentiation of epithelial cells toward a mesenchymal condition (EMT) is a complex process that allows tumor cells to migrate to ectopic sites. Cadherins are not just structural proteins, but they act as sensors of the surrounding microenvironment and as signaling centers for cellular pathways. However, the molecular mechanisms underlying these signaling functions remain poorly characterized. Cadherin-6 (CDH6) is a type 2 cadherin, which drives EMT during embryonic development and it is aberrantly re-activated in cancer. We recently showed that CDH6 is a TGF β target and an EMT marker in thyroid cancer, suggesting a role for this protein in the progression of this type of tumor. Papillary thyroid carcinomas (PTCs) are usually indolent lesions. However, metastatic spreading occurs in about 5% of the cases. The identification of molecular markers that could early predict the metastatic potential of these lesions would be strategic to design more tailored approaches and reduce patients overtreatment. In this work, we assessed the role of CDH6 in the metastatic progression of thyroid cancer. We showed that loss of CDH6 expression profoundly changes cellular architecture, alters the inter-cellular interaction modalities and attenuates EMT features in thyroid cancer cells. Using a yeast two-hybrid screening approach, based on a thyroid cancer patients library, we showed that CDH6 directly interacts with GABARAP, BNIP3 and BNIP3L, and that through these interactions CDH6 restrains autophagy and promotes re-organization of mitochondrial network through a DRP1-mediated mechanism. Analysis of the LIR domains suggests that the interaction with the autophagic machinery may be a common feature of many cadherin family members. Finally, the analysis of CDH6 expression in a unique cohort of human PTCs showed that CDH6 expression marks specifically EMT cells, and it is strongly associated with metastatic behavior and worse outcome of PTCs.

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INTRODUCTION

Thyroid tumors are the most common malignancies of the endocrine system.¹ Papillary thyroid carcinomas (PTCs) account for almost 90% of the thyrocyte-derived cancers. PTCs are usually indolent lesions characterized by a slow rate of growth and a low-metastatic potential. Distant metastases arise in < 5% of patients and survival rate is over 90% (over 98% in young patients) in the first 10 years after diagnosis.^{2,3} In spite of the low-risk profile, all patients who are diagnosed with PTCs are treated with invasive and aggressive approaches.^{4,5} Thus, a major clinical challenge is currently to distinguish in the early stage of diagnosis patients who need aggressive treatments to reduce mortality from those who do not. Identification of markers that are specifically associated with the aggressive behavior of thyroid tumor cells should help overcome this hurdle.

The epithelial-to-mesenchymal transition (EMT) is a multistep process through which the epithelial cells shed their differentiated characteristics to undergo changes in morphology, architecture, adhesion and motility.^{6–8} The ultimate result of this process is to confer migration properties to tumor cells. Adhesion molecules like cadherins have a crucial role in EMT. Cadherins are a family

of transmembrane proteins, the major functions of which are to dictate the modalities of cell interaction with the microenvironment, and to mediate the organization of cytoskeleton and the structural architecture of the cells.⁹ Biophysical forces applied by cell–cell or cell–ECM adhesion are translated into the cells by cadherins, and mediate the organization of cytoskeleton and the architecture of the cells. Beside this structural function, cadherins have a more complex role, acting as chemical and biophysical sensors of the surrounding microenvironment and as signaling centers for pathways controlling cell growth, fate and behavior.^{10–14} The molecular mechanisms underlying the signaling function of cadherins are still poorly characterized.

Cadherin-6 (CDH6) is a class II cadherin involved in the morphogenesis of central nervous system and kidney.^{15–18} As many proteins involved in embryogenesis, CDH6 is aberrantly re-activated in cancer.^{19,20} Recently, we found CDH6 as strongly expressed in a model of highly aggressive thyroid cancer, and we showed that CDH6 expression is induced by TGF β during EMT in thyroid cancer cells.^{21,22} These observations suggest that CDH6 is part of a program that supports aggressiveness of thyroid tumors. In this work, we investigated the biological role of CDH6 in thyroid

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cancer cells and the molecular mechanisms through which this protein partake to thyroid cancer progression.

RESULTS

Silencing of CDH6 inhibits cell proliferation and induces changes in cellular morphology and architecture

In order to define the function of CDH6 in thyroid cancer, we targeted its expression with small-interference RNA (siRNA) in two different thyroid cancer cell lines (TPC1 and BCPAP) (Supplementary Figure 1A–D). In both models the cellular proliferation, measured by growth curve and MTT assay, was profoundly inhibited upon CDH6 abrogation (Figure 1a–d). In accordance, analysis of cell cycle showed that CDH6 silencing induced a reduction of G1/S transition (Figure 1e and f). Silencing of CDH6 also repressed cell migration in a wound-healing assay and invasion (Supplementary Figure 1E–G). Strikingly, suppression of CDH6 expression dramatically changed cell morphology and cell–cell interaction properties (Figure 1g and h). Under reflectance light microscopy, CDH6 silenced cells displayed a flat shape and an increased cell size as compared with control cells that instead retained morphology of the parental cell line. Furthermore, while the cell boundaries were evident in siCTRL cells, contacts between adjacent cells were no longer distinguishable in siCDH6 cells that appear to be like a uniform layer of fused cells. Moreover, the staining of cell membranes with β -catenin highlighted a striking roof tile-like organization of siCDH6 cells (Supplementary Figure 2A and B) that was not detected treating cells with control siRNA (siCTRL).

This observation seems to suggest that loss of CDH6 expression changes cellular architecture of thyroid cancer and alters the inter-cellular interaction modalities. Next, we analyzed the effect of CDH6 silencing on the architecture of cytoskeleton by staining the actin filaments using a fluorophore-conjugated phalloidin. We observed that the density of membrane protrusions (like lamellipodia and filopodia) was reduced in siCDH6 as compared with control cells (Figure 2a and b). Furthermore, in siCTRL cells the actin filaments appeared to be localized at the edge of the cells running parallel to the cell membrane, whereas silencing of CDH6-induced actin assembly into thick fibers (stress fibers) spanning across the cytoplasm along the major axis, and interconnecting cells at the level of cell junctions. This kind of thick and stable actin fibers have been previously described as a feature of non-motile cells and to be different from the stress fibers normally associated with highly motile cells that are instead thinner, shorter and structured into a more chaotic organization.²³

We also analyzed the arrangement of focal adhesions upon CDH6 silencing by assessing the phosphorylation status of FAK and the localization of p-FAK (phosphorylated-FAK). Following CDH6 silencing, p-FAK was displaced from the cell membrane and recruited along the actin fibers, in particular at the nucleation center of the fibers (Figure 2c and d). Furthermore, the overall amount of p-FAK decreased in siCDH6 cells (Figure 2e). This effect on the cell–cell junction organization was confirmed by the observation that, upon CDH6 silencing, the localization of the tight junction protein ZO1 changed from a uniform cell-surface distribution to a focal localization at the level of the cell–cell contacts, in association with the actin fibers (Figure 2f). ZO1 protein levels were not changed (Figure 2g). Intriguingly, a recent work describes that CDH6 is required for neural crest cells migration and detachment during hindbrain development. In accordance with our data, these authors show that ablation of CDH6 results in altered spatiotemporal dynamics of F-actin filaments and in particular in the loss of apical accumulation of actin filaments, which is a necessary event for cell migration.²⁴

CDH6 silencing attenuates EMT phenotype in thyroid cancer cells. Recently, we described CDH6 as a marker of EMT in thyroid cancer²² and we showed here that its expression changes cell–cell interaction. Thus, we investigated whether silencing of CDH6 affects epithelial–mesenchymal differentiation. First, we measured by western blot analysis the expression of E-cadherin (ECAD) and N-cadherin (NCAD), which are respectively markers of epithelial and mesenchymal phenotype in EMT (Figure 2g). NCAD protein expression was consistently downregulated upon silencing of CDH6 in both cell lines. By contrast, TPC1 and BCPAP cells do not express ECAD and silencing of CDH6 did not alter this state. Furthermore, we evaluated the expression of Cadherin16 (CDH16) by qRT-PCR (Figure 2h). CDH16 is a well-established epithelial marker of thyroid cells, which is strongly repressed in thyroid carcinoma²⁵ and it has been described as marker of MET in renal fibrosis where its expression is negatively regulated by SNAIL.²⁶ CDH16 mRNA expression was significantly induced upon silencing of CDH6. On the contrary, no effect was observed on the mRNA levels of NCAD and ECAD. This suggests that the decreased protein expression of NCAD observed in siCDH6 cells is determined by reduced protein stability. All together, these observations indicate that, even if not sufficient to fully revert EMT, silencing of CDH6 attenuates EMT features in thyroid cancer cells.

CDH6 interacts with GABARAP and restrains autophagy

To further characterize CDH6 function at the molecular level, we used a yeast membrane two-hybrid screening to identify CDH6-interacting proteins. To this purpose, CDH6 full-length protein was used as bait to screen a cDNA expression library constructed from five human PTC samples. The library was normalized to subtract part of the most abundant cDNAs, increasing the possibility to identify less-abundant CDH6 interactors. Twelve CDH6-interacting proteins were identified. GABARAP was the most frequently isolated CDH6 interactor in this analysis (Supplementary Figure 3a). BNIP3L, GATE16 and BNIP3 were also identified. GABARAP and GATE16, homologs of the yeast ATG8 protein, are ubiquitin-like modifiers that, during autophagy, are covalently conjugated to membrane lipids to induce formation of the autophagosome.²⁷

BNIP3 and BNIP3L are multifunctional mitochondrial proteins, involved in apoptosis and mitochondrial dynamics.²⁸ Noticeably, GABARAP/GATE16 are known to form functional complexes with BNIP3/BNIP3L to mediate the autophagic degradation of malfunctioning mitochondria.²⁹ The direct interaction of CDH6 with GABARAP, GATE16, BNIP3 and BNIP3L was assessed by GST pull-down experiments (Figure 3a). Interaction with CDH6 was confirmed for GABARAP, BNIP3 and BNIP3L, but not for GATE16. Autophagy is a basic catabolic mechanism that has been implicated in cancer progression, both with a tumor promoting or tumor-restraining function.³⁰ Thus, we sought to analyze the functional consequences of the interaction between CDH6 and GABARAP in thyroid cancer cells. LC3 is the most representative marker of autophagy that, together with GABARAP, is lipidated to induce the autophagosome formation and it is degraded after the fusion with lysosome. Western blot analysis showed that silencing of CDH6 in TPC1 cells induced a significant decrease of the endogenous levels of LC3 (Figure 3b) and a significant decrease in the level of Flag-GABARAP (Figure 3c). Chloroquine is an autophagy inhibitor that blocks the fusion of autophagosomes with lysosomes and the degradation of the autophagosome cargo. Chloroquine treatment blocked the siCDH6-induced decrease of LC3 levels (Figure 3d), suggesting that this effect is determined by LC3 increased consumption during the autophagosome digestion rather than other autophagy-unrelated processes. To confirm that siCDH6 affects autophagy in thyroid cancer cells, we analyzed the activation status of LC3 and GABARAP upon CDH6 silencing (Figure 3e and f). In control cells, both proteins showed a diffuse

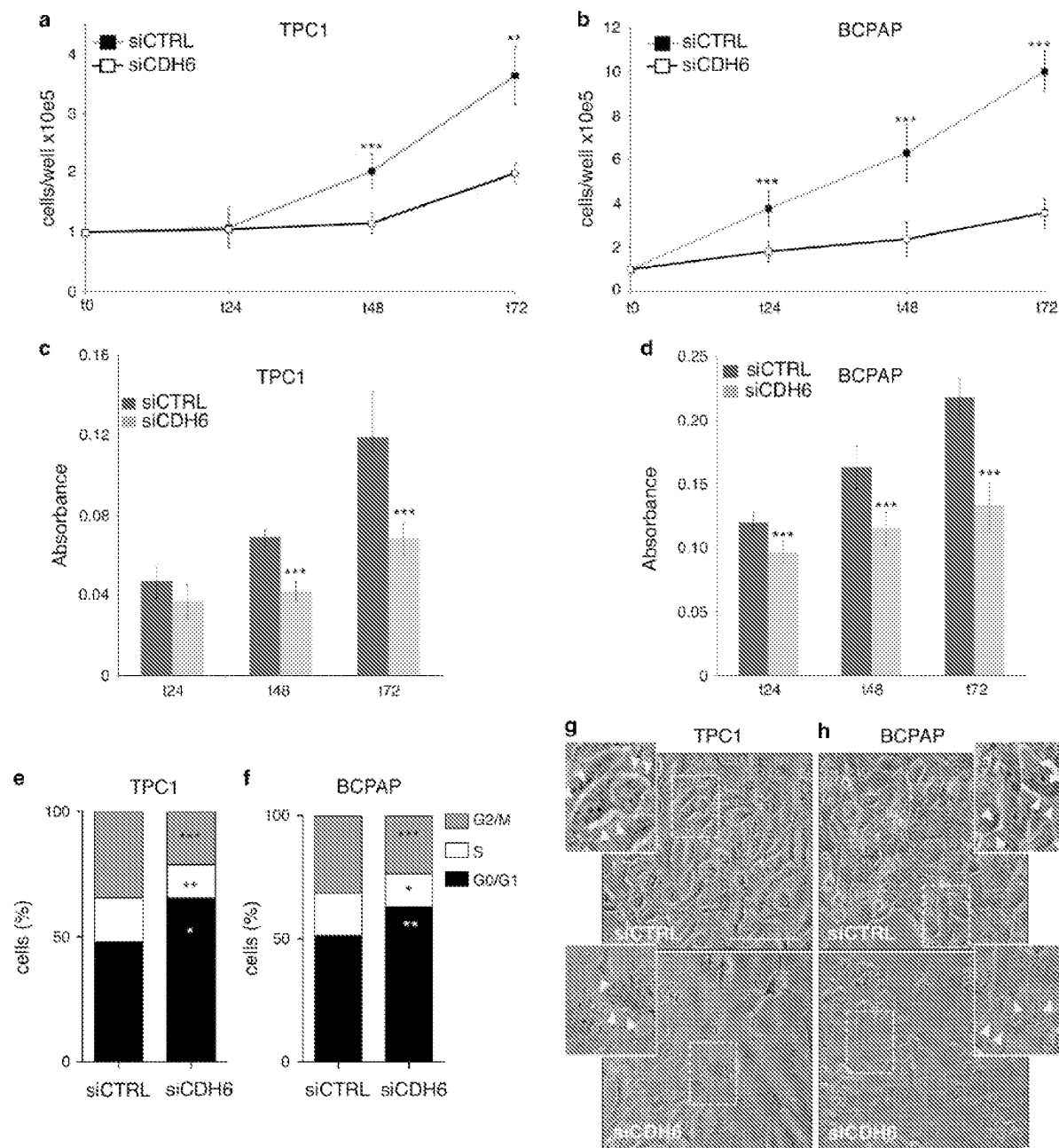


Figure 1. Silencing of CDH6 inhibits cell proliferation and induces profound changes in cell morphology. **(a, b)** Proliferation curves of TPC1 **(a)** and BCPAP **(b)** cells transfected with CDH6 or siCTRLs. Curves represent the average number of cells per well $\pm$ s.d. (four replicates for points) for each transfection at each time point. The entire experiment was repeated three times obtaining consistent results. **(c, d)** MTT-based vitality assay in TPC1 **(c)** and BCPAP **(d)** cells transfected with CDH6 or siCTRLs. The bars represent the average value (six replicates) of absorbance at 490 nm after blank subtraction and correction for absorbance at 620 nm $\pm$ s.d. The entire experiment was repeated three times obtaining consistent results. **(e, f)** Cell cycle analysis in TPC1 **(e)** and BCPAP **(f)** cells transfected with CDH6 or siCTRLs. The histograms represent the average respective proportion of G0/G1 phase, S-phase and G2/M phase of siCDH6 and control groups $\pm$ s.d. obtained in three different experiments. **(g, h)** Light microscope imaging of siCTRL and siCDH6 TPC1 and BCPAP cells 96 h after transfection. Arrowheads indicate cell boundaries. Scale bars, 100 μ m. * P < 0.05, ** P < 0.01, *** P < 0.001.

and homogeneous staining across the cytoplasm. By contrast, in siCDH6 cells both LC3 and GABARAP were mainly localized into clear dots indicating that silencing of CDH6 induced LC3 and GABARAP lipidation and activation. Immunofluorescence staining

confirms that LC3 and GABARAP co-localize in the same structures upon silencing of CDH6 (Figure 3g). The inhibition of the PI3K/AKT/mTOR axis is one of the leading mechanisms driving autophagy activation.³⁰ Indeed, p-AKT levels significantly

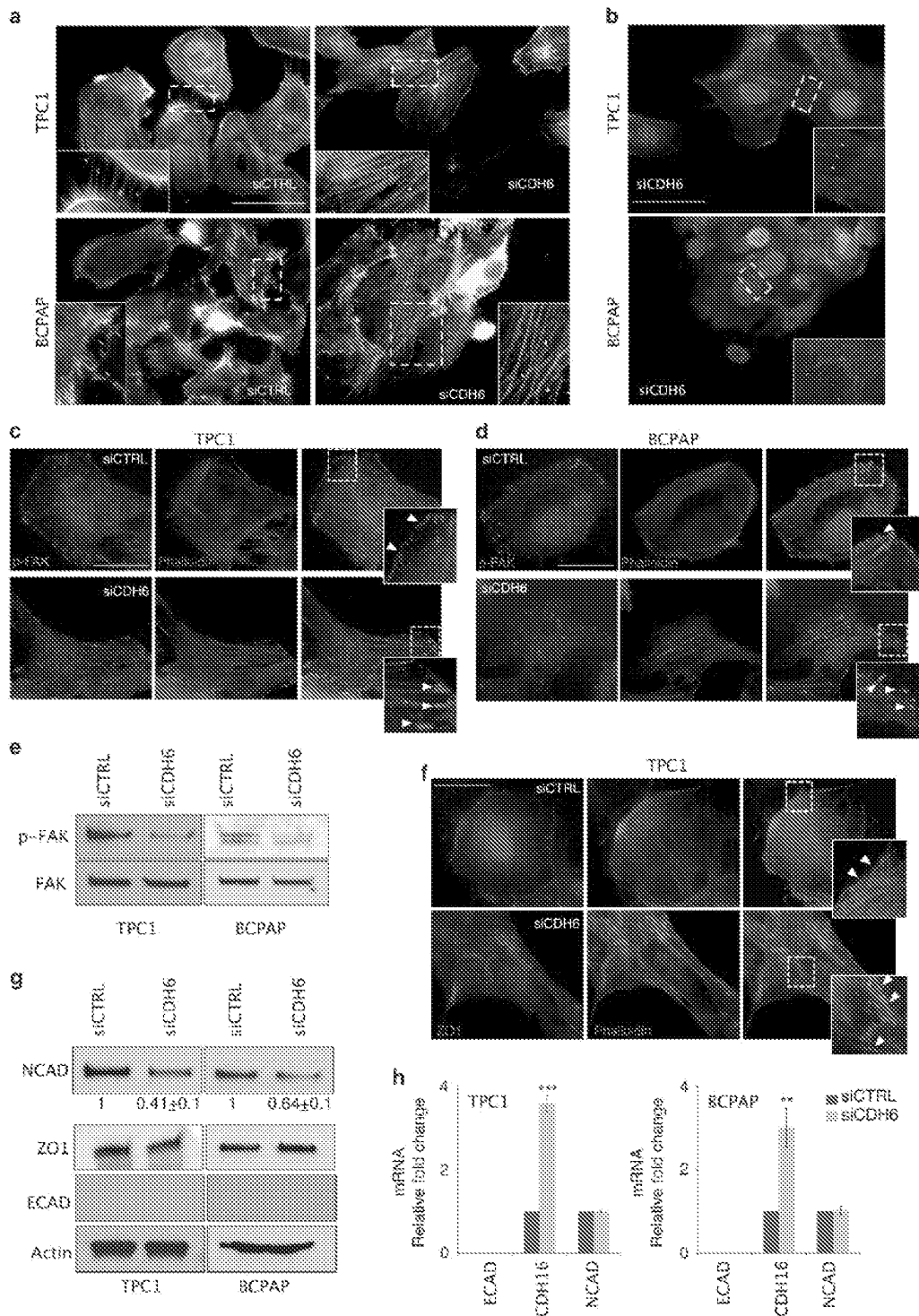


Figure 2. Silencing of CDH6 affects cellular architecture and EMT in PTC cells. (a, b) Immunofluorescence staining of actin filaments (Phalloidin). (c, d) Immunofluorescence staining of actin filaments and p-FAK. (e) Western blot analysis of total and p-FAK. (f) Immunofluorescence staining of actin filaments and ZO1. (g) Western blot analysis of ZO1, NCAD and ECAD in TPC1 and BCPAP cells. Quantification indicates the average percentage of NCAD normalized on the Actin levels in siCDH6 cells as compared with siCTRL cells $\pm$ s.d. (no. of replicates = 3). (h) qRT-PCR analysis of CDH16, ECAD and NCAD. Scale bars, 100 μ m (a, b); 50 μ m (c, d, f). * P < 0.05, ** P < 0.01, *** P < 0.001.

decreased in siCDH6 as compared with control cells (Figure 3h) in accordance with the increased lipidation of LC3 and GABARAP. By contrast, only marginal effects were observed on ERK phosphorylation (Figure 3i) upon silencing of CDH6. Massive activation of autophagy affects mitochondrial dynamics and induces mitochondrial fusion.³¹ In turn, mitochondrial organization may affect cytoskeleton and cell motility.³² Analysis of mitochondria by fluorescent staining showed that silencing of CDH6 induced a massive re-organization of mitochondria into a highly structured and interconnected tubular network (Figure 3j and k). These structural changes were associated with a significant down-regulation of exogenous BNIP3 and of the endogenous DRP1, which is the master driver of mitochondrial fission³³ (Figure 3l and m). qRT-PCR analysis did not show significant alterations in the expression of other mitochondrial dynamics controlling genes, with the exception of PINK1, a kinase known to target dysfunctional mitochondria to the autophagic degradation, which was upregulated in siCDH6 cells (Supplementary Figure 3B). Even if further experiments are needed these evidences suggest that CDH6 expression increase DRP1 and BNIP3 protein stability.

Next, we showed that overexpression of the cytoplasmic domain of CDH6 is not sufficient to mirror the phenotype observed in the CDH6 loss-of-function experiments (Supplementary Figure 4), indicating that CDH6 full-length protein, and likely its membrane localization (Supplementary Figure 5), is required for CDH6 function in the cytoskeleton organization and for its autophagy-restraining activity. Unfortunately, we were not able to obtain overexpression of CDH6 full-length protein in either of the two thyroid cancer cell lines.

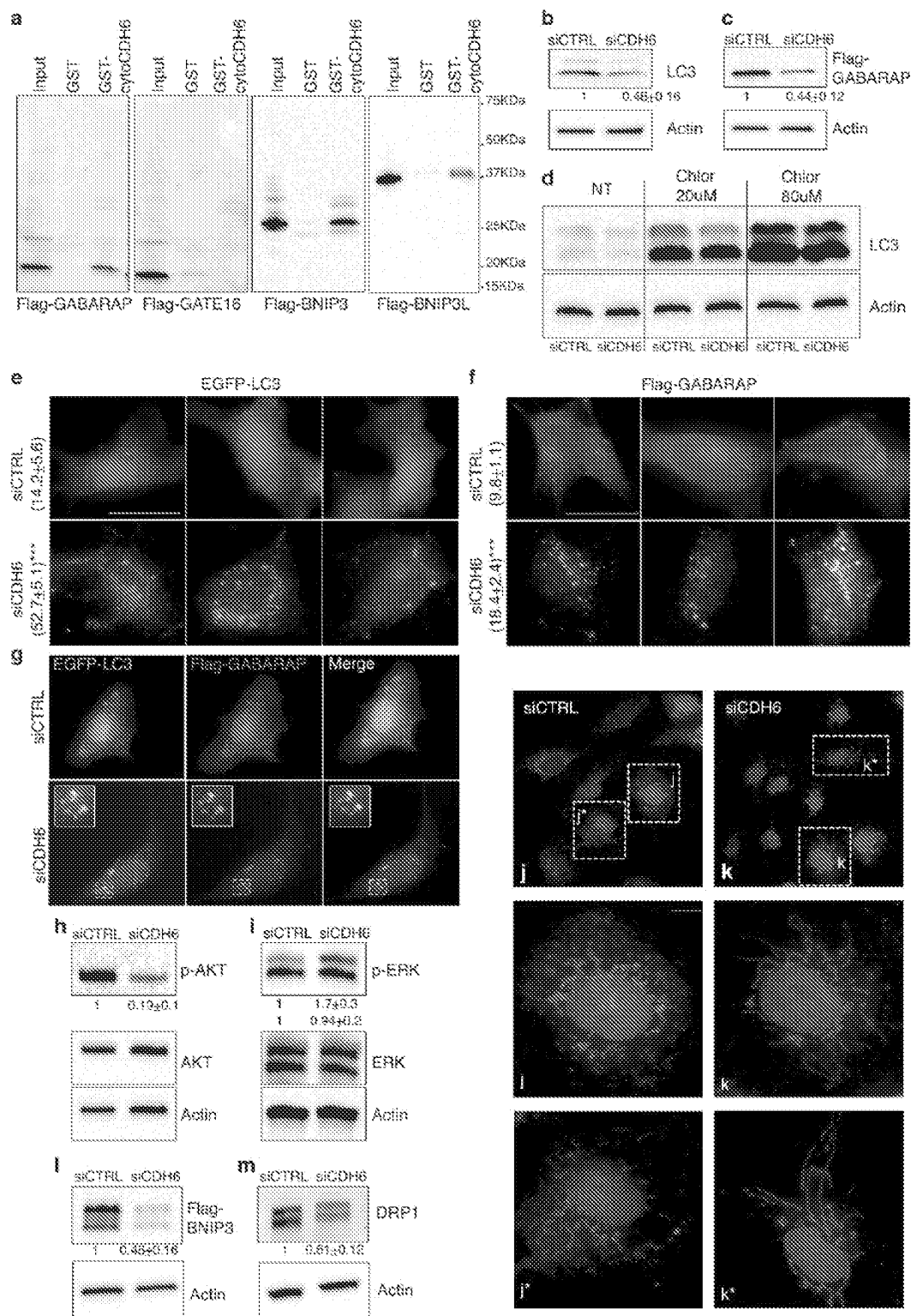
Non-redundant LIR domains are required for the binding of CDH6 to the autophagic machinery

The selective interaction of proteins with the autophagic machinery is mediated by a short linear motif named LIR domain.³⁴ CDH6 cytoplasmic region (cytoCDH6) contains 4 putative LIR domains (LIR1–4; Figure 4a). We deleted each of the predicted LIR domains and used a GST pull-down approach to define their role (Figure 4b) in binding with CDH6 interactors. Deletion of LIR2 abolished the binding of cytoCDH6 with GABARAP. In contrast, deletion of LIR3 increased the affinity of cytoCDH6 to GABARAP, suggesting structural interference among these sites. Intriguingly, deletion of LIR3 impaired the binding of cytoCDH6 with BNIP3 and BNIP3L. These observations indicate that at least two of the four LIR motifs are necessary for the interaction of CDH6 with the autophagic machinery and that they are not functionally redundant. Further analyses are needed to fully understand the structure of these interactions, however, these data seem to indicate that CDH6 interacts with the autophagic apparatus with a structural epitope rather than a single linear motif (as described for the rest of LIR containing proteins). Next, we explored the conservation of the four LIR domains among the other cadherins. Eighteen of the 23 cadherins contain a cytoplasmic domain. Protein sequence alignment of the cytoplasmic regions of these cadherins showed a striking conservation of LIR1, 2 and 3 across the members of the family (Figure 4c), whereas LIR4 was present only in CDH6. This observation indicates that the binding to GABARAP, BNIP3 and BNIP3L may be a common feature of the cadherin family. Of note, the epithelial marker CDH16, which expression is induced upon silencing of CDH6 in thyroid cancer cells, has a truncated cytoplasmic domain thus lacking all the four LIR domains. This seems to suggest that the switch between CDH16 and CDH6 is functionally relevant to support EMT in thyroid cancer and indicate an additional mechanism (beside the changes in extracellular interactions) through which cadherin switch may mediate EMT during tumor progression.

CDH6 expression is associated with distant metastasis and decreased survival in PTC patients

The clinical feature mainly associated with a negative outcome of PTC patients is the presence of distant metastasis at the time of diagnosis. To confirm the hypothesis that CDH6 is important in defining the aggressive potential of PTCs, we evaluated CDH6 expression and its association with the development of distant metastasis in human well-differentiated PTCs by immunohistochemistry. Owing to the low aggressiveness of this type of tumor, metastatic spreading is a rare event in PTCs, which takes place in < 5% of all cases.¹ We searched the thyroid tumor archive of our institution over the past 30 years (counting over 2700 cases), and we retrieved a very large cohort of 44 well-differentiated PTCs that develop distant metastasis (DM-PTCs). To the best of our knowledge this represents one of the largest cohort of well-differentiated PTCs with distant metastasis ever characterized at the molecular level.³⁵ As control, we selected 42 PTCs that did not develop distant metastasis based on a minimum follow-up of 7 years (Supplementary Table 1). Noticeably, we observed a striking correlation between CDH6 expression and the presence of distant metastasis (43.2% of DM-PTCs; 16.7% of control PTCs; $P=0.001$; Table 1). As well, we observed that CDH6 expression was associated with a reduced disease-free survival probability in the overall cohort (Figure 5a), but not in the DM-PTCs group (Figure 5b), suggesting that CDH6 may be relevant specifically in the early phases of the metastatic spreading of thyroid cancer. None of the other morphological and clinical variable taken into consideration showed significant correlation with CDH6 expression (Supplementary Table 1). The expression of CDH6 was not homogeneous within the tumor tissue but confined to restricted foci of cells localized at the invading front of the tumor (Figure 5c–e). Not only DM-PTCs showed a higher frequency of CDH6 positivity, but also had a much higher percentage of CDH6 expressing cells as compared with control PTCs (Table 1). Of note, in DM-PTCs the CDH6-positive cells displayed a mesenchymal-like phenotype with an elongated shape and less-structured cell-cell interaction as compared with the rest of the tumor cells (Supplementary Figure 6A–D), resembling the peculiar morphological changes of EMT. In all the analyzed cases, CDH6-positive cells form small clusters embedded in the connective tissue surrounding the tumor and retain ECAD expression (Figure 5c–e). This organization strongly resemble a well-known phenomenon named 'collective cell migration' which has been indicated as instrumental for epithelial cancer invasion and metastatic spreading.^{36–39} During collective cell migration, cells undergo a partial EMT, changing morphology toward a spindle-like phenotype but retaining some epithelial features including ECAD expression, which is required to allow cohesion during migration.^{40,41} In our analysis ECAD was strongly and homogeneously expressed in the set of PTC analyzed and its expression did not correlate with the presence of distant metastasis or with other parameters taken into consideration in this study (Supplementary Tables 2 and 3). Loss of ECAD expression has been shown to occur in the highly aggressive and poorly differentiated forms of thyroid cancer.^{42,43} Because of the diverse nature of the cohort in analysis (well-differentiated PTCs, with or without distant metastasis), the high levels of ECAD expression were expected and do not appear to be in conflict with previous findings.

Intriguingly, when we analyzed the correlation of CDH6 expression with the clinical outcome only within the DM-PTCs group, we observed that CDH6 expression was not associated with a worse course of the disease (Figure 5b), suggesting that CDH6 may be relevant specifically in the early stages of thyroid cancer metastatic spreading, in accordance with its potential role in the EMT process. Indeed, once tumor cells have reached the metastatic site, the mesenchymal features acquired through the



EMT have to be reverted (through the opposite mesenchymal-to-epithelial transition process) in order to allow metastatic cells to proliferate and colonize the site.⁵

DISCUSSION

Cadherins are transmembrane proteins, the major function of which is to define structure and organization of the cell interactions with the surrounding microenvironment.^{9,14} Several observations indicate that this structural function is only a limited part of a more complex role that cadherins have in the regulation

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of cell biology. However, these additional functions remain poorly characterized at the level of molecular mechanisms.

In this work we provide evidences for a previously unknown role of CDH6 in controlling autophagy and mitochondrial dynamics in thyroid cancer.

We showed that:

1. CDH6 expression is a marker of high metastatic potential in human thyroid cancer.
2. CDH6 controls cellular architecture and dictates the structure of the cell-cell interaction.

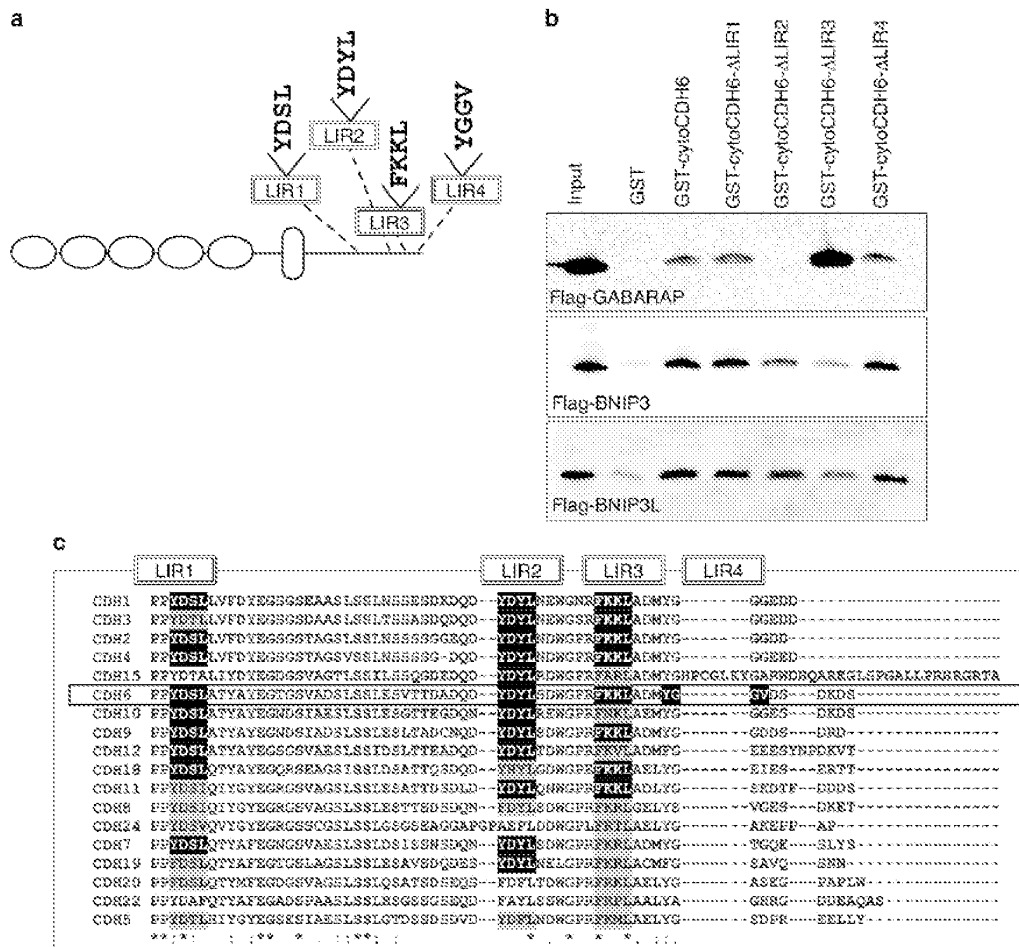


Figure 4. CDH6 contains multiple functional LIR domains. **(a)** Representation of cytoCDH6 LIR domains. **(b)** GST pull-down analysis of CDH6-LIR mutants with the identified interactors. This is a representative experiment of three replicates. **(c)** LIR1-4 conservation in the cytoplasmic regions of cadherins family members.

Figure 3. CDH6 restricts autophagy and controls mitochondrial dynamics. **(a)** GST pull-down analysis of cytoCDH6 binding to the identified interactors. A representative experiment of four replicates is shown. **(b, c)** Western blot analysis of endogenous LC3 and exogenous Flag-GABARAP in TPC1. Quantification indicates the averaged percentage of LC3 and Flag-GABARAP normalized on the levels of actin in siCDH6 cells as compared with siCTRL cells $\pm$ s.d. (no. of replicates = 3). **(d)** Western blot analysis of LC3 in siCTRL and siCDH6 in untreated or Chloroquine-treated TPC1. **(e, f)** Immunofluorescence analysis of localization of EGFP-LC3 and Flag-GABARAP. Quantification indicates number of dots/cell $\pm$ s.e.m. **(g)** Immunofluorescence staining showing co-localization of EGFP-LC3 and Flag-GABARAP upon silencing of CDH6. Western blot analysis of p-AKT, AKT **(h)**, p-ERK and ERK **(i)** in TPC1. Quantification indicates the averaged percentage of p-AKT and p-ERK normalized on the levels of total AKT, ERK in siCDH6 cells as compared with siCTRL cells $\pm$ s.d. (no. of replicates = 3). **(j, k)** Immunofluorescence staining of mitochondria in TPC1. Western blot analysis of exogenous Flag-BNIP3 **(l)** and endogenous DRP1 **(m)** in TPC1. Western blot quantification represents the relative averaged value of three independent experiments $\pm$ s.d. Scale bars, 100 μ m.

Table 1. CDH6 expression in PTC cases

	0 (negative)	1 ($< 5\%$ positive cells)	2 (5–10% positive cells)	3 ($> 10\%$ positive cells)	χ^2 -test
DM-PTC	25 (0.57)	0 (0.0)	7 (0.16)	12 (0.27)	$P = 0.001$
Control-PTC	35 (0.83)	2 (0.05)	5 (0.12)	0 (0.0)	
LNM-PTC	19 (0.95)	0 (0.0)	1 (0.05)	0 (0.0)	$P = 0.13$
noMet-PTC	16 (0.73)	2 (0.09)	4 (0.18)	0 (0.0)	
Total	60 (0.70)	2 (0.02)	12 (0.14)	12 (0.14)	

Abbreviations: DM-PTC, papillary thyroid cancer with distant metastases; LNM-PTC, PTC with local lymph node metastases; noMet-PTC, PTC without any metastases.

- CDH6 expression promotes the acquisition of some mesenchymal features.
- CDH6 restrains autophagy and promotes DRP1-dependent mitochondrial fission.

On the basis of these observations we propose the following model (Figure 5f): CDH6 expression is turned on by pro-metastatic signals (like TGF β). Expression of CDH6 alters the cellular architecture loosening the tightness of cell–cell interactions and favoring a cytoskeleton organization suitable to sustain the mechanical stress of cell invasion. In parallel, CDH6 restrains autophagy promoting DRP1-dependent mitochondrial fission through the interaction with GABARAP and BNIP3 and/or BNIP3L. This generates profound changes in the structure of mitochondrial network increasing the availability of fixed mitochondria, which in turn are necessary to sustain and promote the re-organization of actin cytoskeleton and the formation of structures of movement. As a result of this multifunctional activity, CDH6 promotes EMT, collective cell migration and metastasization of thyroid cancer. Even if further experiments are needed, this model is in accordance with the previously described activity of CDH6 in EMT^{22,24} and is supported by a consistent number of works reporting an inhibitory function of autophagy on the activation of the EMT program,⁴⁴ and a relevant role of the DRP1-dependent mitochondrial fission in promoting cancer migration and aggressiveness.^{45–48}

EMT and autophagy are linked in a controversial and still undefined relationship. Although EMT promotes autophagy to support viability (through induced resistance to anoikis and immune surveillance escape) of potentially metastatic cancer cells,^{44,49,50} autophagy acts to negatively control EMT and activation of the autophagic machinery has been shown to determine a reversion of the EMT phenotype, majorly by reducing the stability of key EMT proteins. As well, we observed that the induction of autophagy promoted by CDH6 silencing is associated with a partial reversion of the EMT phenotype and it is associated with a reduced stability of the mesenchymal marker NCAD, in thyroid cancer cells. Our data suggest that the interaction with GABARAP is central for the restraining effect of CDH6 on autophagy. However, the interaction between these two proteins could have additional functional consequences. GABARAP, which for long time has been studied for its function in the central nervous system as partner of the GABA receptors, has been shown to interact with both microtubules and actin filaments and, through this interaction, to control cytoskeleton dynamics⁵¹ and membrane proteins clusterization on the cell surface.⁵² Thus, it is likely that GABARAP directly partakes to the CDH6-dependent cytoskeleton organization and polarization.

It has been shown that a massive activation of autophagy induces mitochondrial fusion³¹ and that an highly interconnected mitochondrial network inhibits cell migration.³² Vice versa,

increased mitochondrial fission and loss of mitochondrial network have been described as features of oncogenic transformation³³ and increased cancer aggressiveness.^{45,47} Furthermore, the expression of DRP1, the master regulator of mitochondrial fission, is upregulated in metastatic as compared with non-metastatic cancer cells.^{44,46} On the basis of our results, we hypothesize that CDH6 influences mitochondrial dynamics blocking autophagy and stimulating mitochondrial fission through DRP1 expression. The resulting fragmentation of the mitochondrial network would in turn provide the cells with a generous pool of free mitochondria across the cytoplasm, a preferred substrate for cytoskeleton re-organization, lamellipodia formation and cell migration.

In this work, we mainly focused on the interplay between CDH6 and GABARAP, but we provided preliminary evidences that also the binding of CDH6 with BNIP3/BNIP3L could be a relevant part of this mechanism, even if further experiments are needed.

Noticeably, a recent work showed that BNIP3 supports melanoma cell migration by changing the cytoskeleton organization and the cell–cell interaction.⁵³ In particular, silencing of BNIP3 in melanoma cells determines inhibition of cell proliferation, changes in cell morphology and a profound re-organization of actin cytoskeleton, characterized by a re-localization of actin filaments from the cell edge and their organization into thick fibers extending from the cell surface through the cytoplasm. This phenotype strongly resembles the one we observed in thyroid cancer upon CDH6 silencing and suggests that CDH6 or another tissue-specific cadherin cooperates with BNIP3 in determining this phenotype.

It is now well-accepted that cadherins are not just structural proteins, but they function as sensors of external environmental stimuli and as signaling centers for several cellular pathways. The high degree of conservation of the LIR domains across cadherins suggest that other members of this family beside CDH6 have similar role in controlling autophagy and mitochondrial dynamics. Even if further experiments are required, our data provide a new biological function of cadherins and open new perspectives to fully understand the mechanism of action of these proteins both during embryonic development, and in the processes leading to cancer progression.

In this work, we described for the first time CDH6 as a potential marker of distant metastasis in human well-differentiated PTCs. Differently from the highly aggressive poorly differentiated forms of thyroid cancer, the well-differentiated PTCs develop distant metastasis only in a very small percentage of cases (2–5% after 10 years). Because of this low frequency and the long latency of progression, very little is currently known about the molecular mechanisms that drive aggressiveness and metastasization of these tumors. In this context, we propose a potential model for PTC progression that rely on the crucial role of CDH6 and on the interplay between EMT and autophagy.

MATERIALS AND METHODS

Patients selection and immunohistochemistry

Eighty-six well-differentiated PTCs were retrieved from the archive of the Pathology Unit of the Arcispedale S. Maria Nuova, that comprises over 2700 thyroid cancers collected in the past 30 years, including over 1800 PTCs. Tumor slides were screened by 2 authors (SP and MR) and classified according to the criteria recommended by the World Health Organization Classification of Tumors to exclude high-grade carcinomas, and staged according to the American Joint Committee on Cancer Staging Manual, seventh edition. Among the overall PTCs, 44 PTCs that developed distant metastases outside the neck were identified. In 36% of the DM-PTCs the distant metastases were present at the time of diagnosis (synchronous), whereas in 64% of DM-PTCs, distant metastases arose later (metachronous). Site of distant metastases is reported in Supplementary Table 1. As control sample, we selected 42 PTCs that did not develop distant metastases, based on a follow-up of 7 years, matched by age with DM-PTCs cases. All the patients underwent total thyroidectomy, and in 80% of

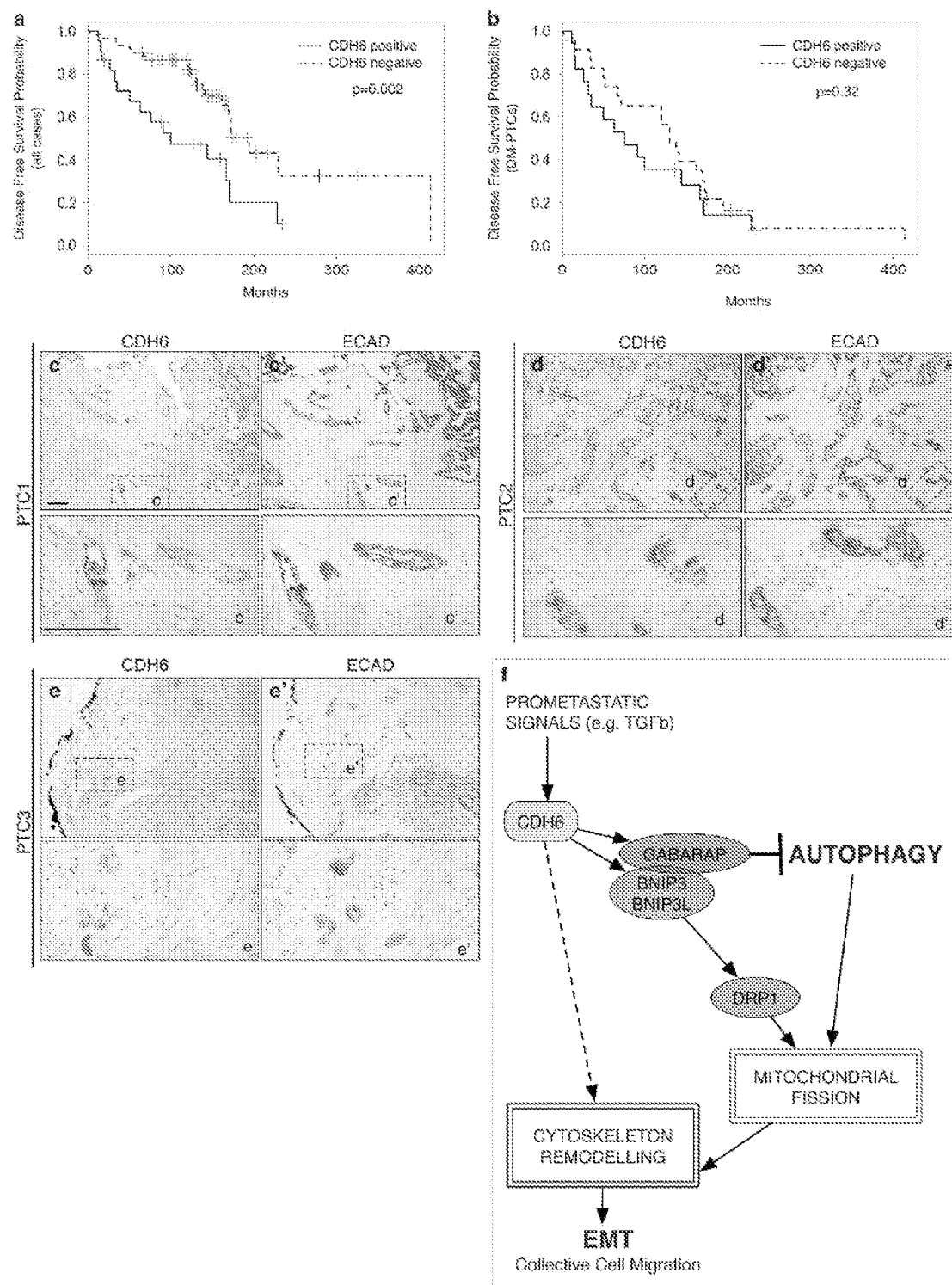


Figure 5. CDH6 expression is associated with distant metastasis in PTCs. (a, b) Kaplan-Meier curves of disease-free survival probability by CDH6 status in the overall PTC population ($n=86$, (a)) or in the DM-PTC population ($n=44$, (b)). (c-e) Representative immunohistochemistry analysis of CDH6 and ECAD in three different DM-PTCs. (c-e) represent higher magnification of inserts in c-e. Scale bars 100 μ m. (f) Model of CDH6 function in thyroid cancer cells.

the cases, they also underwent ipsilateral central neck dissection. Follow-up for all cases ranged from 13 to 372 months. Immunohistochemistry was performed as previously described²⁴ using a monoclonal anti-CDH6 antibody (HPA007047) from Sigma Aldrich (Milan, Italy), and the monoclonal anti-ECAD antibody (clone 36) (790-4497) from Ventana-Roche (Monza, Italy). For immunohistochemistry on TPC1, BCPAP and MCF7 (Supplementary Figures 1 and 5) cells were harvested and fixed in formalin for 10 min. After centrifugation the cells were resuspended in 0.6% agar in phosphate-buffered saline 1× to be included in paraffin. Five micron sections were stained as tissue samples. Supplementary Figures 1C and D, S and 7A–C show the specificity control for the staining.

This project has been approved by the Arcispedale S. Maria Nuova of Reggio Emilia ethics committee and informed consent was obtained from the patients.

Cell cultures and transfection.

BCPAP and TPC1 PTC cell lines were obtained from Dr Santoro, University of Naples (Naples, Italy). BCPAP that overexpress *Id1* were clonally derived from BCPAP upon transfection with an *Id1* overexpressing vector as previously described.^{21,22} Both cell lines were grown at 37 °C and 5% CO₂ in DMEM supplemented with 10% fetal bovine serum. All cell lines were authenticated by SNP profiling at Multiplexion GmbH (Immenstaad, Germany); dates of last authentication reports are 9 December 2014, and 12 November 2014. Stealth RNA interference oligos against CDH6 and control oligos were purchased from Thermo Fisher Scientific (Monza, Italy). In all, 20 nm of either anti-CDH6 or control oligos were transfected using the RNAiMax Lipofectamine reagent (Thermo Fisher Scientific) using the reverse-transfection protocol. With the exception of cell morphology analysis (Figure 1g and h) for which the cells were analyzed after 96 h, the rest of functional experiments were performed 48 h after siRNA transfection. To minimize possible biases owing to different cell density, we transfected the cells with siRNA at day 0 in a regular plate. After 32 h the cells were harvested and seeded at the same cell density (siCDH6 and siCTRL cells) either in 24 well-plates (for western blot and RNA analysis) or on slide chambers for immunofluorescence experiments. Cells were analyzed the morning after (about 12 h after seeding and 48 h after siRNA transfection).

For co-transfections of siRNA oligos and plasmids, 48 h after siRNA transfection, siCDH6 or siCTRL cells were transfected with the indicated plasmids using Lipofectamine 2000 (Thermo Fisher Scientific). Twenty-four hours after plasmid transfection, cells were processed for Western blot or immunofluorescence.

Yeast Two-Hybrid screening

The yeast two-hybrid screen was done by DualSystems Biotech AG (Schlieren, Switzerland), using the split-ubiquitin system for membrane proteins. This approach requires the construction of a bait by fusing a membrane protein of interest to the C-terminal half of ubiquitin (Cub) and a transcription factor. The prey is constructed by fusing a cDNA library (including both membrane proteins and cytosolic proteins) to the N-terminal half of ubiquitin (NubG). An interaction between bait and prey results in assembly of split-ubiquitin from Cub and NubG. Proteases recognize the split-ubiquitin and cleave the transcription factor from the bait. The transcription factor moves to the nucleus and activates the reporter genes in the yeast genome, converting the interaction at the membrane into a reporter readout. Full-length cDNA encoding human CDH6 was cloned into the pBT3-SUC vector and used as bait. For generation of the prey, total RNA was extracted from 5 PTC samples selected from the Head and Neck Tumors Biobank of our institution. The RNA from these samples was pooled and used to generate a normalized cDNA library in the pPR3-N vector. Library normalization consists of removal of high-abundance transcripts, ensuring good representation of low-abundance transcripts in the library and reducing the rate of false positives. The complexity of the cDNA library was 2.8×10^6 independent clones with an average size of 1.5 kb. The bait was transformed together with the cDNA library into yeast host strain NMY22. A total of 3.8×10^6 transformants were screened, which yielded 39 positive clones. The library plasmids were isolated from positive clones, and the identity of positive interactors was determined by sequencing. DNA sequences were translated and searched against the Swissprot database using the BLASTX algorithm.

GST pull-down

For GST and GST fusion proteins purification, BL21 *E. coli* (New England Biolabs, Ipswich, MA, USA) were transformed with pGEX-6P-1, pGEX-6P-1_cytoCDH6 or pGEX-6P-1_cytoCDH6-UR1-4 and were grown overnight. The day after they were diluted 1:100 in 50 ml of LB with 100 mg/ml Ampicillin (Sigma Aldrich, Milan, Italy) and grown until optical density at 600 nm reached 0.6–0.8. The expression of recombinant proteins was induced with 0.4 mM IPTG (Sigma Aldrich) for 2 h. Cells were pelleted and lysed in phosphate-buffered saline 1× plus 1% Triton X-100 (Sigma Aldrich) and sonicated. Supernatants were incubated with 50 µl of 50% slurry Glutathione Sepharose 4B (GE Healthcare, Milan, Italy). After three washes in phosphate-buffered saline 1×, the bound proteins were eluted in Protein Sample Buffer (Biorad, Segrate, Italy) and the expression of GST or GST fusion proteins was checked by SDS-PAGE using Stain Free gels (Biorad). GABARAP, GATE16, BNIP3 and BNIP3L were transcribed and translated *in vitro*, in fusion with the FLAG epitope, using the TnT T7 Coupled Reticulocytes Lysate System (Promega, Milan, Italy) following the manufacturer's protocol. Protein expression was checked by SDS-PAGE and western blot using the anti-FLAG antibody (Sigma Aldrich). For the interaction, Glutathione Sepharose beads bound to 1 µg of GST, GST-cytoCDH6 or GST-cytoCDH6-UR-mut1-4 recombinant proteins were incubated with each CDH6 interactor expressed *in vitro* for 2 h at 4 °C in Interaction Buffer (20 mM Hepes, 150 mM KCl, 0.02% NP-40, 0.02 mg/ml BSA, 2 mM EDTA, 2 mM DTT plus Complete Protease Inhibitor Cocktail (Roche, Monza, Italy)). Beads were washed in Interaction Buffer and bound proteins were eluted in Protein Sample Buffer (Biorad) for SDS-PAGE and Western blot analysis using anti-Flag antibody.

Statistical analysis

Statistical analyses were performed using R Statistical Software package (R Foundation for Statistical Computing, Vienna, Austria; <http://www.R-project.org>). Frequencies in immunohistochemical staining classes were compared between groups by χ^2 -test. Clinicopathological parameters of patients were reported as frequencies or as mean $\pm$ s.d., and were compared between immunohistochemical staining classes by χ^2 -test and *t*-test. Survival curves were plotted by the Kaplan–Meier method with the log-rank statistics.

For all the *in vitro* experiments, results are reported as mean $\pm$ s.d. unless otherwise specified. Number of technical replicates within each experiment and number of experiments conducted for each analysis is indicated in the corresponding figure caption. Assumptions of each statistical test were verified. *P*-values < 0.05 were considered as threshold for significance.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Supplementary Information: Additional information on this paper on the Oncogene website (<http://www.nature.com/onc>)

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Antibody Drug Conjugates for Cancer Therapy

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Abstract—Antibody drug conjugates (ADCs) constitute a family of cancer therapeutics designed to preferentially direct a cytotoxic drug to cells expressing a cell-surface antigen recognized by an antibody. The antibody and drug are linked through chemistries that enable release of the cytotoxic drug or drug adduct upon internalization and digestion of the ADC by the

cell. Over 40 distinct ADCs, targeting an array of antigens and utilizing a variety of drugs and linkers, are undergoing clinical evaluation. This review primarily covers ADCs that have advanced to clinical investigation with a particular emphasis on how the individual targets, linker chemistries, and appended drugs influence their behavior.

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I. Introduction

Most reviews on antibody drug conjugates (ADCs) begin by citing Paul Ehrlich's prophetic "magic bullet" proposal from 1908 (Strebhardt and Ullrich, 2008). In actuality, though, magic is an illusion, whereas the ragged, convoluted path that has led to the still incremental success of antibody drug conjugates today is painfully real. If one could administer a therapeutically effective dose of a highly pan-cytotoxic compound to a living organism without evoking toxicity, that would indeed be magic. So far, one cannot. It is fanciful to expect the pan-toxic compound to enter a cell of one type, in this case cancer, and kill it, but enter a cell of another type, such as liver, skin, or intestine, and remain benign. The idea that appending such a compound to a vehicle specifying its delivery would ameliorate said toxicity is highly intriguing, yet equally specious. The fact remains that only a very small portion of the ADC actually enters the intended target cell while the remainder goes elsewhere. That elsewhere includes normal tissues that catabolize the conjugate, releasing the active compound to wreak havoc on the catabolizing cell, as well as neighboring cells. The liberated drug can also diffuse into general circulation, resulting in systemic exposure to distant tissues.

That normal tissues lacking target are impacted by ADCs should not be too surprising. Antibodies exhibit extensive half-lives *in vivo*, but they do not last forever. Nor do they specifically home to their intended targets after systemic administration. The distribution and ultimate catabolic fate of antibodies has been shown to occur throughout the body in a variety of tissues (Henderson et al., 1982; Wright et al., 2000; Garg and Balthasar, 2007). The nonspecific pinocytotic uptake of ADCs into normal cells residing in these tissues may differ from target-dependent uptake, but in either case they are ultimately routed to the lysosome for degradation, whereupon the drug is unleashed. Although a monumental effort has been rightfully allotted to the identification of tumor-specific targets and the development of stable drug linkers, target-independent toxicity resulting from normal turnover of the antibody continues to vex the advancement of ADCs.

Although a forthright discussion regarding the current limitations of ADCs is warranted, significant advances have occurred, and with more on the horizon, there remains great cause for optimism. Two ADCs, Kadcyla (trastuzumab emtansine; Genentech, South San Francisco, CA) and Adcetris (brentuzumab vedotin; Seattle Genetics, Seattle, WA), have been Food and Drug Administration approved and enjoy widespread use in

the oncology clinic (Jackson and Stover, 2015). These ADCs prove that the therapeutic index of an otherwise untenable cytotoxin can be elevated to a therapeutically beneficial level by affixing it to an antibody. Kadcyla and Adcetris are not without side effects, though, as thrombocytopenia and neuropathy, respectively, can limit their dosing, thus underscoring the need for further improvements. Because the therapeutic index of an ADC is a function of several components, there exist several entry points for potential improvements (Fig. 1). The choice of target, the antibody, the chemistry and site of drug attachment, and the nature of released drug all influence the risk benefit ratio of ADCs. Here I will attempt to address each of these components by outlining examples of their implementation and how they impact the behavior of ADCs.

II. The Drugs Conjoined With Antibodies

A. Doxorubicin

Some of the earlier iterations of ADCs tested in the clinic included the incorporation of antimitotics and antimetabolites approved for use in chemotherapy as unconjugated cytotoxins (Ford et al., 1983; Oldham et al., 1988; Elias et al., 1990; Petersen et al., 1991; Krauer et al., 1992; Takahashi et al., 1993). These early clinical ADCs suffered from a panoply of issues, not the least of which was a drug likely too impotent for targeted delivery. These studies also preceded the broad application of humanized or human therapeutic antibodies and reported frequent hypersensitivity reactions to the administered mouse monoclonal antibodies. Premature release of drug from the antibody also contributed to a loss of efficacy and, in some cases, toxicity. A subsequent iteration, again using a standard chemotherapeutic, doxorubicin, largely circumvented the hypersensitivity reactions with a human/mouse chimeric antibody. This immunconjugate, referred to as BR96-doxorubicin, targeted the Lewis Y antigen and carried up to eight doxorubicin molecules per antibody conjugated through an acid labile linker (Hellstrom et al., 2001). Because of a lack of efficacy, BR96-doxorubicin failed to progress beyond a phase II metastatic breast cancer trial (Tolcher et al., 1999). A teachable element here was the significant difference in the nature of the toxicities—hematopoietic toxicity with free doxorubicin and gastrointestinal toxicity with the ADC. It was surmised that Lewis Y expressed in the gut was responsible for the contrasting toxicities. However, as discussed later in this review, toxicities not typically associated with a free drug can arise when it is conjugated to an antibody, irrespective of the antibody target.

ABBREVIATIONS: ADC, antibody drug conjugates; AML, acute myeloid leukemia; CanAg, cantuzumab mertansine; DAR, drug-to-antibody ratio; EphA2, Eph family receptor tyrosine kinase A2; HER2, human epidermal growth factor receptor II; HL, Hodgkin's lymphoma; MC, maleimidocaproyl; MMAE/F, monomethylauristatin E/F; MSLN, mesothelin; mTG, microbial transglutaminase; NHL, non-Hodgkin's lymphoma; PBD, pyrrolobenzodiazepine; SMCC, succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate; SPDB, N-succinimidyl 4-(2-pyridyldithio)butyrate; SPP, N-succinimidyl 4-(2-pyridyldithio)pentanoate; SS, disulfide; VC, valine-citrulline.

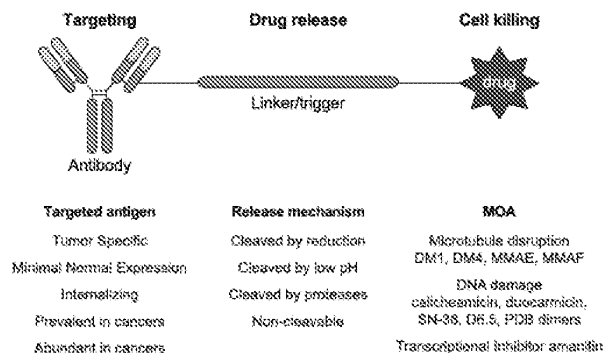


Fig. 1. The three primary ADC components that determine which cells are targeted (antibody), how the drug is released (linker/trigger), and the mechanism of action (MOA) by which cells are killed. All indicated linker/triggers and drugs, except amanitin, are under clinical investigation as ADCs.

B. Calicheamicin

The requirement for increased potency was met head on by conjugating the highly cytotoxic compound calicheamicin to a humanized antibody recognizing CD33 (Hinman et al., 1993). Calicheamicin is a bacterially derived antibiotic that binds somewhat specifically to oligopyrimidine-oligopurine sequences in the minor groove of DNA, leading to double strand scission. Calicheamicin exhibits cell killing potency that can exceed that of standard of care antimitotics by 1000-fold (Lee et al., 1987; Zein et al., 1988). A slightly less potent, but more stable, derivative of calicheamicin, *N*-acetyl- γ -calicheamicin dimethyl hydrazide, was used for incorporation into ADCs (Fig. 2). A calicheamicin ADC, gemtuzumab ozogamicin, targeted CD33 and was investigated for the treatment of relapsed and newly diagnosed acute myelocytic leukemia (AML) in a variety of single agent and combination clinical trials. Initial response rates appeared encouraging and led to market approval, but subsequent studies revealing excessive toxicity and a lack of improved overall survival when used in combination with standard of care therapy resulted in withdrawal from the market (Jurcic, 2012). Nevertheless, a retrospective meta-analysis uncovered an overall survival benefit in AML patients with favorable cytogenetics prompting an evaluation of gemtuzumab ozogamicin in that setting (Loke et al., 2015). A second ADC containing calicheamicin, inotuzumab ozogamicin, and targeting CD22, has been investigated in hematologic malignancies. Although a Phase III trial in relapsed/refractory non-Hodgkin lymphoma failed to reach its endpoint, encouraging activity has been observed in earlier stage trials of relapsed/refractory acute lymphoblastic leukemia (Shor et al., 2015).

C. Auristatins and Maytansinoids

Currently, the most widely implemented cytotoxins in the development of ADCs in the clinic are derived from

the natural product dolastatin 10, originally isolated from a sea hare and later found to be synthesized by cyanobacteria ingested by it (Pettit et al., 1987; Luesch et al., 2002). Comprised of a 4-mer peptide of unconventional amino acids capped with a C-terminal dolaphenine (Fig. 2), dolastatin 10 disrupts microtubules and kills cells with a potency 20–50 times greater than that of vinblastine (Bai et al., 1990). Several clinical trials investigating the antineoplastic activity of dolastatin 10, and a synthetic derivative TZT-1027, were performed but objective responses were not achieved (Singh et al., 2008a). Further derivatives of dolastatin 10, containing substitutions at the C-terminal dolaphenine with norephedrine or phenylalanine, yielded auristatins E and F, respectively (Maderna et al., 2014). Omission of a methyl group from the N terminus afforded the respective monomethyl analogs monomethylauristatin E/F (MMAE and MMAF, respectively), containing a secondary amine amenable to chemical derivatization with linkers for conjugation to antibodies (Doronina et al., 2003). These two compounds, predominantly MMAE, are the most common cytotoxic agents employed in ADCs currently under clinical investigation (Table 1). One of the MMAE conjugates, Adcetris (brentuximab vedotin), received approval by the Food and Drug Administration in 2011 for the treatment of Hodgkin lymphoma and anaplastic large cell lymphoma (Younes, 2014).

Like dolastatin 10, maytansine was originally isolated as a natural product that displayed highly potent cytotoxic activity resulting from its ability to disrupt microtubule polymerization (Kupchan et al., 1972; Lopus et al., 2010) (Fig. 2). However, a paucity of objective responses in the cancer clinic hampered further development of maytansine as a chemotherapeutic (Ravry et al., 1985; Cassady et al., 2004). Interest in its antineoplastic activity was reinvigorated by the incorporation of maytansinoid thiol derivatives into antibody drug conjugates (reviewed by Lambert, 2013). Two maytansine derivatives, termed DM1 and DM4, differing primarily by the degree of methylation on the carbon atom adjacent to the disulfide bond (Kellogg et al., 2011), are undergoing clinical investigation as ADC warheads attached to various antibodies (Table 1). One of the DM1 ADCs, ado-trastuzumab emtansine (Kadcyla), targeting the human epidermal growth factor receptor II (HER2) showed remarkable activity in HER2-positive metastatic breast cancer in a Phase III clinical trial and was approved for use in this indication (Verma et al., 2012). The HER2 homolog HER1, commonly referred to as EGFR, has also been targeted by an ADC incorporating DM1 and is in early stage clinical testing.

D. Pyrrolobenzodiazepine

Although maytansines and auristatins, both of which disrupt microtubule dynamics, dominate the ADC clinical landscape, additional early stage clinical studies have been initiated with ADCs containing highly

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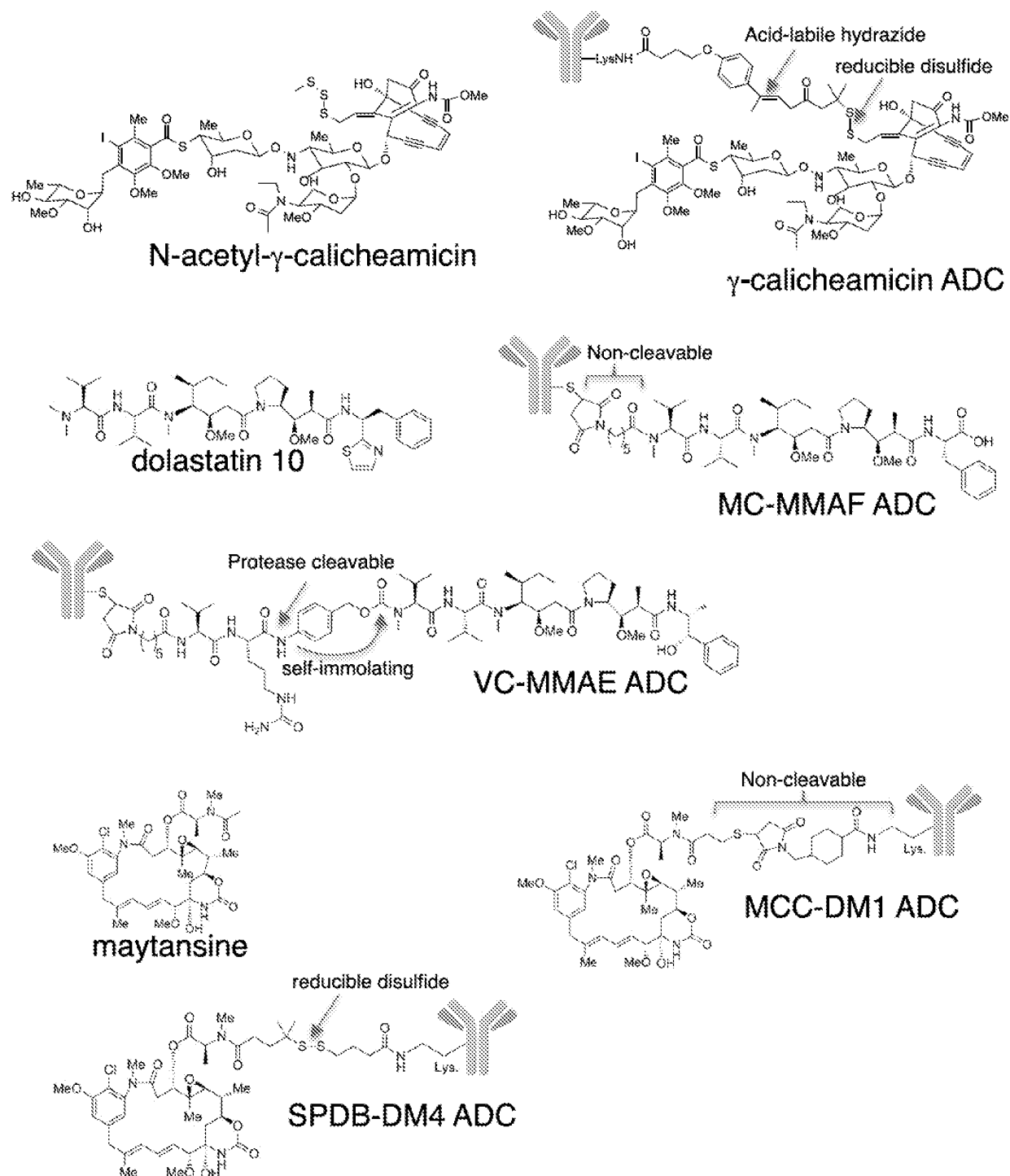


Fig. 2. Structures of the drugs and their derivatives used in ADCs. Linkers and drug release mechanisms (red lines) are illustrated in the context of the ADC.

potent DNA damaging agents. In particular, a synthetic dimerized derivative of pyrrolobenzodiazepine (PBD), an anthramycin class of antibiotic conjugated to an antibody targeting CD33 (Kung Sutherland et al., 2013), has recently advanced into the clinic for testing

against acute myeloid leukemia (Stein et al., 2014) (Fig. 2, Table 1). As a free drug, PBD dimers are among the most potent cytotoxic compounds ever identified, exhibiting potency in cell killing assays reaching GI_{50} values in the low to even subpicomolar range (Hartley, 2011).

Antibody Drug Conjugates

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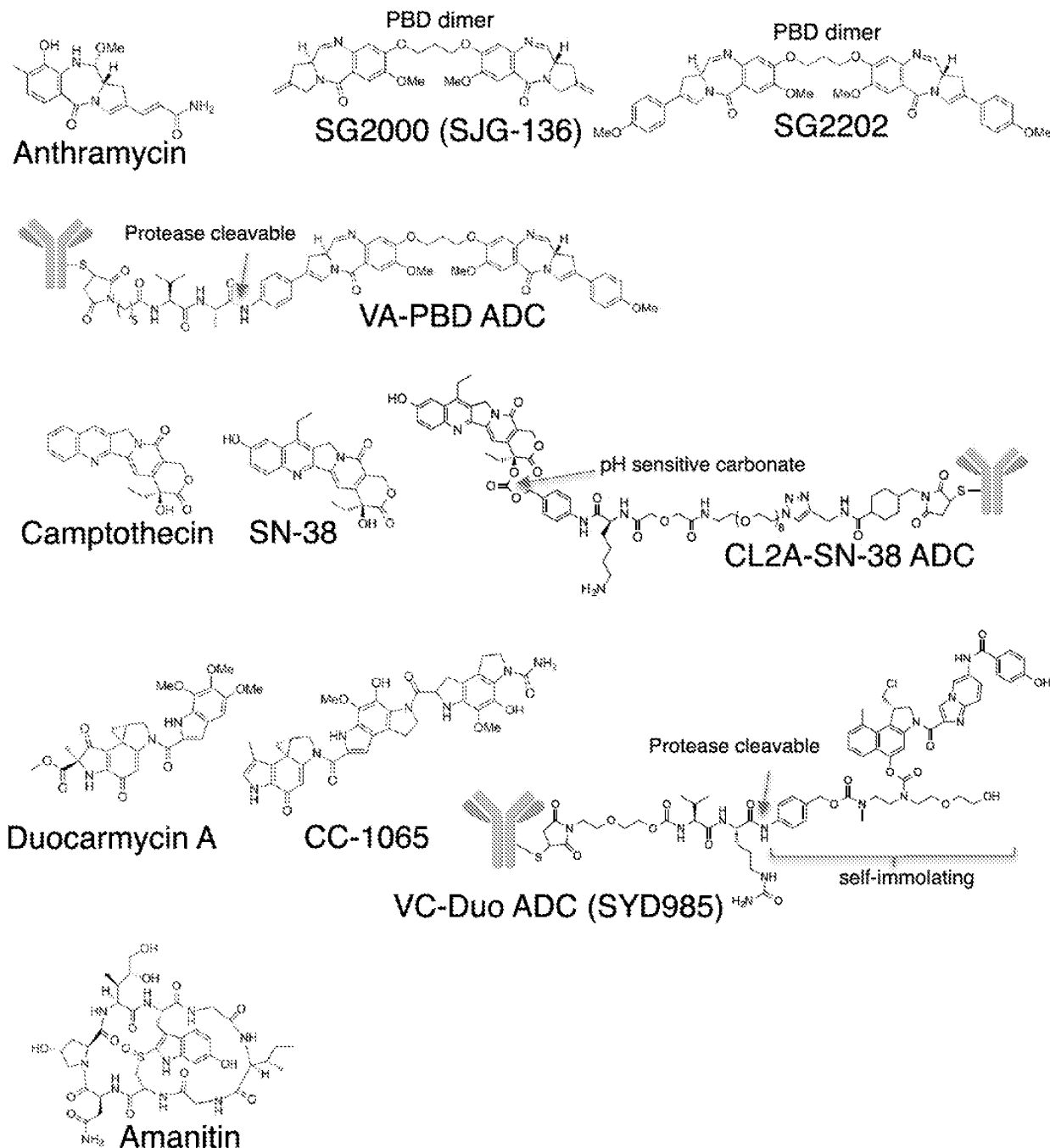


Fig. 2. Continued.

PBD dimers bind in a semisequence selective manner to the minor groove of duplex DNA. Their cytotoxicities are attributed to the formation of adducts to guanine residues on opposing strands of DNA, resulting in interstrand crosslinks (Gregson et al., 2001; Hartley et al., 2004). One of the PBD dimers, SG2000 (SJG-136), entered clinical investigation for the treatment of epithelial ovarian, primary peritoneal, or fallopian tube

cancer, and although some of these studies were terminated, its assessment in advanced chronic lymphocytic leukemia and acute myeloid leukemia currently remain open (ClinicalTrials.gov Identifier: NCT02034227).

E. SN-38

As discussed above, most of the drug components so far employed in the ADCs that have advanced to the

TABLE 1
Clinical investigations of antibody drug conjugates

ADC	Target	Drug	Linker	Indication	Clinical Status (Updated)
Adcetris	CD30	MMAE	VC	HL, ALCL	Approved
DMUC5754A	MUC16	MMAE	VC	ovarian, pancreatic	Phase I: completed (02/2015)
DNIB0600A	NaPi2b	MMAE	VC	ovarian, lung	Phase I/II: recruiting (05/2015)
DSTP3086S	STEAP1	MMAE	VC	prostate	Phase I: recruiting (05/2015)
DCDT2980S	CD22	MMAE	VC	NHL, DLBCL	Phase I/II: recruiting (05/2015)
DCDS4501A	CD79b	MMAE	VC	NHL, DLBCL	Phase I/II: recruiting (05/2015)
DEDN6526A	EDNRB	MMAE	VC	melanoma	Phase I: ongoing (04/2015)
DMOT4039A	MSLN	MMAE	VC	pancreatic	Phase I: ongoing (05/2015)
MLN0264	guanylyl cyclase	MMAE	VC	GI	Phase I/II: recruiting (03/2015)
SGN-LIV1A	LIV1	MMAE	VC	breast	phase I: recruiting (05/2015)
ASG-22CE	NECTIN4	MMAE	VC	solid tumors	Phase I: ongoing (04/2015)
AGS15E	SLITRK6	MMAE	VC	urothelial	Phase I: recruiting (02/2015)
Glembatumumab vedotin	GPVMB	MMAE	VC	TN breast	Phase II: recruiting (06/2015)
PSMA ADC	PSMA	MMAE	VC	prostate	Phase II: completed (03/2015)
HuMax-TF-ADC	tissue factor	MMAE	VC	solid tumors	Phase I: recruiting (11/2014)
SGN-75	CD70	MMAE	VC	NHL, renal	Phase I: completed (12/2014)
ASG-5ME	SLC44A4	MMAE	VC	pancreatic, gastric	Phase I: completed (08/2013)
Bay 79-4620	CA9	MMAE	VC	solid tumors	Phase I: terminated (09/2014)
SGN-CD19A	CD19	MMAF	MC	NHL	Phase I: recruiting (05/2015)
SGN-CD70A	CD70	MMAF	MC	NHL, renal	Phase I: recruiting (05/2015)
AGS-16MF	ENPP3	MMAF	MC	renal	Phase I: completed (12/2012)
PF-0626350	5T4	MMAF	MC	solid tumors	Phase I: recruiting (02/2015)
ABT-414	EGFRvIII	MMAF	MC	solid tumors	Phase I: recruiting (06/2015)
IMGN289	EGFR	DM1	SMCC	solid tumors	Phase I: terminated (06/2015)
AMG 595	EGFRvIII	DM1	SMCC	glioma	Phase I: ongoing (01/2015)
AMG 172	CD27L	DM1	SMCC	renal	Phase I: ongoing (06/2015)
Lorvotuzumab mertansine	NCAM (CD56)	DM1	SPP	heme malignancies	Phase II: recruiting (01/2015)
IMGN388	α v integrin	DM4	SPDB	solid tumors	Phase I: completed (09/2013)
IMGN853	FOLR1	DM4	SPDB	ovarian	Phase I: recruiting (06/2015)
SAR3419	CD19	DM4	SPDB	ALL	phase II: terminated (08/2014)
SAR566658	CA6	DM4	SPDB	solid tumors	phase I: recruiting (05/2015)
BT-062	CD138	DM4	SPDB	multiple myeloma	Phase I: recruiting (05/2015)
BAY-94-9343	mesothelin	DM4	SPDB	solid tumors	Phase I: recruiting (06/2015)
BIIB015	Cripto	DM4	SPDB	solid tumors	Phase I: completed (09/2013)
IMGN529	CD37	DM4	SPDB	NHL	Phase I: recruiting (06/2015)
IMGN853	FOLR1	DM4	SPDB	ovarian	Phase I: recruiting (06/2015)
Inotuzumab oxogamicin	CD22	calich-DMH	SS/hydrazone	ALL	Phase I/II: recruiting (06/2015)
Gemtuzumab oxogamicin	CD33	calich-DMH	SS/hydrazone	AML/PML	Comp. Use: recruiting (08/2014)
SYD985	HER2	seco-DUBA	VC	Her2+ BC	phase I: recruiting (06/2015)
SGN-CD33A	CD33	PBD	VA	AML	phase I: recruiting (05/2015)
SC16LD6.5	fyn3	D6.5	undisclosed	SCLC	Phase I/II: recruiting (04/2015)
Milatuzumab-dox	CD74	doxorubicin	hydrazone	multiple myeloma	Phase I/II: completed (01/2014)
IMMU-130	CEACAM5	SN-38	carbonate	colorectal	Phase I/II: recruiting (01/2015)
IMMU-132	TROP-2	SN-38	carbonate	epithelial	Phase I/II: recruiting (01/2015)

seco-DUBA, seco-duocarmycin-hydroxybenzamide-azaindole; VA, valine-alanine; ALCL, anaplastic large-cell lymphoma; DLBCL, diffuse large B cell lymphoma; TN, triple negative; BC, breast cancer; SCLC, small cell lung cancer; ALL, acute lymphoblastic leukemia; PML, promyelocytic leukemia; CML, chronic myelogenous leukemia.

clinic are not drugs commonly administered as free chemotherapeutic agents. The only exception is an anti-CD74 antibody conjugated to doxorubicin. A second rather atypical example involves the use of SN-38, a derivative of camptothecin, a naturally occurring quinoline alkaloid originally isolated from *Camptotheca*, the Happy Tree (Chazin et al., 2014) (Fig. 2). Although SN-38 is not itself administered as a chemotherapeutic, the prodrug analog, referred to as CPT-11 or irinotecan, is considered standard of care in the treatment of colorectal cancer. Systemic irinotecan is readily converted by human carboxylesterase to SN-38, enhancing its topoisomerase inhibitory activity by over 100-fold relative to irinotecan (Kawato et al., 1991). Two ADCs containing SN-38 and targeting the epithelial cell surface antigens CEACAM-5 and TROP-2, are in Phase I/II clinical studies for the treatment colorectal and triple negative breast cancer, respectively (Govindan et al., 2015; Starodub et al., 2015) (Table 1). Although

the camptothecin analogs promote DNA strand breaks, their mechanism of action differs from calicheamicin and the PBDs, which interact directly with the DNA duplex. Camptothecin analogs, by contrast, cannot bind to DNA alone, but interact with the topoisomerase-DNA complex (Redinbo et al., 1998; Liu et al., 2000) resulting in stalled DNA replication forks.

F. Duocarmycins

An additional class of DNA damaging agents, CC-1065 and the related duocarmycins, has also been applied in ADC technology (Shor et al., 2015) (Fig. 2). These agents interact with the minor groove of duplex DNA and alkylate adenine residues, consequently promoting strand breaks (Boger and Johnson, 1995). A first in human Phase I clinical study of SYD985 was recently opened in which an anti-HER2 antibody conjugated to a duocarmycin is being investigated in HER2-positive breast cancer (ClinicalTrials.gov # NCT02277717). It is

anticipated that this ADC might impact HER2-positive tumors expressing lower levels of HER2 than that associated with the diagnostically positive 3+ tumors targeted by Kadcyla (van der Lee et al., 2015). An additional ADC, MDX-1203, contained an analog of CC-1065 coupled to an antibody targeting CD70 (Thevanayagam et al., 2013). A Phase I clinical investigation of its potential utility in renal cell carcinoma and non-Hodgkin's lymphoma was recently completed (ClinicalTrials.gov # NCT00944905). In this dose escalation study, a relatively high dose of 15 mg/kg was reached, whereupon a dose-limiting toxicity of hypersensitivity in 2 of 16 patients was encountered, but an Maximum Tolerated Dose was not defined (Owonikoko et al., 2014). Additional adverse events included fatigue (85%) and nausea (54%), as well as other common constitutional symptoms, whereas delayed toxicities, described as facial edema and/or pleural or pericardial effusions, were observed in 6/16 (38%) subjects treated at the 15 mg/kg dose. Best response of stable disease was reported for 18 of 26 patients, although it did not correlate with dose level.

G. Other Chemotypes

Preclinical studies describing additional microtubule disrupting agents, such as novel taxoids and tubulysins, that have been conjugated to antibodies also appear interesting (Ojima, 2008; Cohen et al., 2014). Particularly noteworthy is the preclinical assessment of the cytotoxin amanitin as an ADC payload (Fig. 2). Amanitin represents a significant deviation from the microtubule disrupting and DNA damaging agents most commonly used in ADCs. Derived from poisonous mushrooms, amanitin is an octamer of cyclized amino acids that binds to mammalian RNA polymerase II with high affinity, thereby disrupting DNA transcription and causing cell death (Cochet-Meilhac and Chambon, 1974). Free amanitin is only poorly diffusible across cell plasma membranes and requires specific organic anion transporters, with restricted tissue expression, to facilitate its transport into cells (Letschert et al., 2006). Thus conjugating amanitin to an antibody results in highly specific toxicity to cells bearing the antibody target antigen. A comparison of free amanitin toxicity to that of an amanitin ADC targeting Epithelial Cell Adhesion Molecule (EpCAM), resulted in IC_{50} values that were up to 10,000-fold lower on EpCAM-positive cell lines relative to free amanitin (Moldenhauer et al., 2012). In vivo antitumor activity was also apparent at doses considerably lower than that which induced toxicity in mice.

In closing this section, it should be noted that a primary distinction between microtubule disrupting drugs, such as the auristatins and maytansines, and some of the aforementioned DNA damaging agents, is the increased propensity of the latter drugs to kill nonproliferating cells (Shor et al., 2015). This may be

viewed as an advantage in the context of treating indolent cancers or exterminating so-called cancer stem cells that may undergo slow rates of cell division. However, the destruction of normal stem cells, as well as any nonregenerating cell types, particularly endothelial cells, could yield unacceptable toxicities.

III. Antibody-Drug Linkers

A. Stability and Drug Release

The term "stability" is frequently bandied about when discussing ADCs, where it typically refers to retention of drug by the antibody either *ex vivo* in buffers, plasma, or blood or *in vivo* after administration. However, the antibody itself can be destabilized by drug conjugation, resulting in faster clearance of total antibody postdosing (Fig. 3A). Finally, the term "stability" can also apply to the ultimate liberation of drug upon cellular uptake and catabolism of the ADC. For the sections here on drug linkers and their sites of attachment, discussions on stability will primarily refer to extracellular release of drug from antibody.

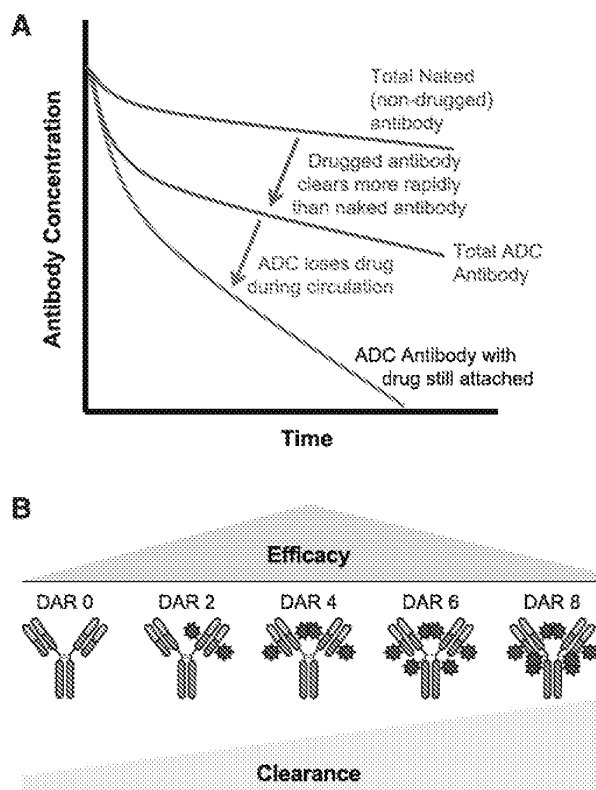


Fig. 3. (A) Illustration depicting two types of pharmacokinetic instability of an ADC. Conjugating an antibody with drug can accelerate its clearance (green versus blue line). Loss of drug from the ADC during circulation results in less drugged antibody relative to total antibody (blue versus black line). (B) Conceptualized relationship between drug antibody ratio (DAR), antitumor activity (efficacy), and elimination of antibody from blood (clearance). Excessive drugging can lead to a loss of efficacy due to reduced ADC exposure.

The rapid release of drug after administration of the ADC seems counterintuitive with respect to fulfilling the "magic bullet" prophecy. Accordingly, the evolution of antibody-drug linker chemistry was largely guided by the desire to maintain stability during systemic circulation followed by release upon internalization of the ADC into cancer cells. In many respects, the ADC linker tenets echo the general ambitions of prodrug therapy, aptly described by Singh et al. (2008b), "Thus, the major objective of prodrug design is to temporarily alter the physicochemical properties of drugs to accomplish modification of drug pharmacokinetics, prolongation of action, reduce toxicities and side effects, increased selectivity, and resolve formulation challenges." Upon internalization, the ADC is routed to the lysosome, where in contrast to plasma, the highly hydrolytic environment is acidic and replete with proteases. Accordingly, acid labile and protease susceptible linkers were implemented (Hamann et al., 2002; Doronina et al., 2003). The high levels of reducing equivalents in the cell cytoplasm, relative to that in plasma, have also been exploited by using linkers with reducible disulfide bonds (Chari et al., 1992). These three modalities of drug release—proteolysis, reduction, and pH-catalyzed hydrolysis—account for all of the ADCs in clinical testing in which cleavable linker technology is employed (Table 1).

B. Noncleavable Linkers

Quite surprisingly, it is not always necessary to release the drug from the amino acid it is appended to in the antibody. Kadcyla, approved for use in human cancer, is prepared using the crosslinking agent succinimidyl 4-(*N*-maleimidomethyl) cyclohexane-1-carboxylate (SMCC), leaving the nonreducible thioether MCC linker positioned between antibody lysine residues and DM1 (Fig. 2). With this linker, the resulting drug remains attached to a lysine residue upon internalization and digestion of the antibody in the lysosome yet retains its ability to kill cells (Erickson et al., 2010). In the case of Kadcyla, preclinical efficacy was comparable to that observed with the anti-HER2 ADC containing maytansinoids coupled through reducible linkers (Lewis Phillips et al., 2008). A noncleavable linker, maleimidocaproyl (MC), is also used with the auristatin ADCs in which MMAF is the indicated cytotoxin (Table 1). Here, the resulting drug retains the linker plus a cysteine residue derived from the antibody (Doronina et al., 2006). It should be noted that although the MCC and MC linkers described here do not contain a cleavable bond, a retro-Michael reaction involving the maleimide moiety is possible and can result in deconjugation of drug from antibody in vivo or in other matrices (Alley et al., 2008; Pillow et al., 2014).

A common feature of the noncleavable linkers used for DM1 and MMAF is that the released drug derivatives are hydrophilic and thus not very potent when added to intact cells (Doronina et al., 2006; Erickson et al., 2010).

This suggests that the free drug released from cells expressing antibody target may be less impactful to neighboring cells not expressing the target. This "bystander effect," or lack thereof, could have implications for treating tumors heterogeneously expressing the target. This was borne out in preclinical studies using xenograft tumors that homogeneously or heterogeneously expressed the target of maytansinoid ADCs containing the cleavable or noncleavable linkers (Kovtun et al., 2006). Tumors with homogenous expression could be eradicated with either ADC, whereas only the ADC with the cleavable (reducible) linker was effective against heterogeneous tumors. This implies that Kadcyla, which contains the noncleavable linker, does not require bystander killing, perhaps owing to the exceptionally high level of amplified HER2 expression on the selected breast cancers for which it is indicated.

C. The Impact of Linker on Toxicity

The impact of released drug substance on cells lacking ADC target also has obvious safety implications. Despite having comparable efficacy, ADCs with maytansinoids linked through reducible linkers produced greater weight loss in rats than an ADC conjugated via the noncleavable thioether bond (Lewis Phillips et al., 2008). Moreover, in clinical testing, some of the adverse events associated with maytansinoid ADCs appear to be a function of the linker used for drug attachment. For example, thrombocytopenia is a common dose-limiting toxicity associated with Kadcyla, containing a nonreducible bond to DM1 (Krop and Winer, 2014). By contrast, hematologic toxicities were unremarkable with lorvotuzumab mertansine (hu901DM1), containing the highly reducible *N*-succinimidyl 4-(2-pyridyldithio)pentanoate (SPP) linkage to DM1 and targeting CD56. Instead, peripheral neuropathy, unremarkable with Kadcyla, was a common adverse event with lorvotuzumab mertansine (Berdeja, 2014). SAR3419, containing DM4 conjugated through the hindered reducible SPDB linker, also did not evoke serious hematologic toxicity, but instead ocular toxicity was frequently noted (Younes et al., 2012a).

Although these maytansinoid ADCs target different antigens, the target is not likely responsible for the distinct, linker-related, aforementioned toxicities. Other ADCs, sharing the same drug and linker but targeting divergent antigens, exhibit overlapping adverse events. Neuropathy was again a frequent side effect with MLNM2704, containing SPP-DM1 and targeting Prostate Specific Membrane Antigen (PSMA) (Galsky et al., 2008), yet the expression patterns of PSMA and CD56 differ significantly. The neuropathy occurring in response to a cell-permeable tubulin binding agent is not surprising because this is commonly associated with standard of care chemotherapeutic taxanes and vinca alkaloids. Accordingly, one might anticipate peripheral neuropathy with MMAE ADCs

containing cleavable peptide linkers that release cell-permeable free MMAE. This is indeed a common toxicity with the VC-MMAE conjugates, but not frequently noted with the noncleavable MC-MMAF auristatin ADCs, which release a free drug substance with reduced potency on intact cells. Together, this implies that the peripheral neuropathy associated with maytansinoid and auristatin-based ADCs containing cleavable linkers may be a bystander effect resulting from the release of cell-permeable drug products originating from the catabolized ADC.

The ocular toxicity associated SAR3419 is again inconsistent with the expression pattern of the target CD19, which is largely restricted to blood cells but is common to the SPDB-DM4 linker-drug configuration. Similar ocular toxicities, variably described as epitheliopathy, keratitis, dry eye, and blurred vision, have been observed with IMGN-853 and BT062, targeting the folate receptor and CD138, respectively (Jagannath et al., 2011; Kurkjian et al., 2013). Although cleavable through reduction, the SPDB linker is highly stabilized relative to SPP, thereby prolonging retention of drug by the antibody during circulation. Thus, the resulting enhancement to overall exposure of normal tissues, such as eye, to intact ADC could contribute to toxicities not observed with more labile linkers. Grades 1 and 2 eye disorders were also noted for Kadcyla, containing the highly stable SMCC linkage to DM1 (Burris et al., 2011a).

Adverse ocular events are not typically associated with cleavable VC-MMAE ADCs but are noted with auristatin ADCs employing the stable MC-MMAF linker drug configuration (Forero-Torres et al., 2014; Tannir et al., 2014). In the case of the auristatin ADCs, it is more difficult to ascribe the ocular toxicity simply to increased stability of the intact ADC. Despite the lack of a cleavable bond, the MC-MMAF conjugates liberate their drugs in vivo at a rate comparable to their VC-MMAE counterparts (Alley et al., 2008). This is because pharmacological deconjugation occurs through maleimide/thioether fragmentation, resulting in loss of the entire linker-drug structure, a mechanism common to both the cleavable VC- and noncleavable MC-linker auristatin conjugates. Nevertheless, increased overall exposure of the MC-MMAF ADCs may still account for ocular toxicity, because they are typically more tolerable and thus dosed at higher levels than VC-MMAE ADCs. Finally, the enhanced exposure argument may also be consistent with side effects associated with Abraxane (nab-paclitaxel; Celgene, Summit, NJ), a protein-bound form of paclitaxel, relative to free paclitaxel. Interestingly, although ocular toxicities are not characteristic of free paclitaxel, ocular and visual disturbances occurred in 13% ($n = 366$) of patients treated with Abraxane (Package Insert, 12/2014). Overall, this suggests that the ocular events associated with stable ADC linkers are, in part, an outcome of increased

exposures unattainable with less stable linkers that may be limited by other toxicities occurring at lower exposures.

D. Limitations of Linker Stability

The means to achieve near complete stability of the antibody drug bond exist, but whether this is even beneficial remains unclear. As a proof of concept, Alley et al. (2008) incorporated a bromoacetamidocaproyl linker to obtain auristatin-based ADCs that underwent no measurable systemic loss of drug for 2 weeks. However, despite a 25% increase in exposure, relative to the reference ADC containing the maleimidocaproyl linker, no appreciable gain in efficacy was observed. This implies an upper limit to the advantages of increased linker stability. Conversely, a measure of instability may be advantageous in some cases. A requirement for an unstable linker was recently described for IMMU-130, an ADC targeting CEACAM-5 and containing a pH-sensitive carbonate bond to SN-38 (Govindan et al., 2015) (Fig. 2). Nearly half of the eight drugs appended to the antibody were liberated within 20 hours in human serum. Nevertheless, preclinical antitumor activity was demonstrated, albeit with a relatively generous dosing schedule of 25 mg/kg of protein, twice weekly. The requirement for an unstable linker was made evident by testing a similar conjugate containing a systemically stable protease cleavable linker, which proved to be ineffective. This strategy, which results in considerable systemic release of free drug, may be particularly amenable to ADCs containing drugs, like SN-38, that have reduced potencies relative to those used in most ADCs.

IV. Sites of Conjugation

A. Stochastic Conjugation

The vast majority of the ADCs currently under clinical investigation are comprised of linker-drug moieties that are conjugated to either lysine or cysteine residues resident to the native composition of the antibody. The maytansinoids are derivatized with a succinimide ester and reacted with antibody in a specified molar ratio, randomly derivatizing up to 20 different lysines in the heavy and light chain subunits (Wang et al., 2005). The resulting ADC may have an average drug-to-antibody ratio (DAR) of approximately four, whereas any individual molecule may have a DAR ranging from zero to eight (Dere et al., 2013). The auristatins are derivatized with maleimide, which enables a reaction with free thiols, made available by reduction of the cystine disulfide bonds that normally link together the antibody subunits (Doronina et al., 2003). An IgG1 contains four such disulfides, two between heavy-heavy chain and one each for heavy-light chain connections, yielding eight possible free cysteine residues for drug conjugation. Although maximizing the DAR is intuitively tempting, if overloaded

the hydrophobic nature of maytansinoids and auristatins can drive antibody aggregation and negatively impact pharmacokinetics (Hamblett et al., 2004; Chari, 2008). Despite derivatization of all eight interchain cysteine residues with MMAE, these purified ADCs appear identical in size to underivatized native IgG, bind target, and kill cells in vitro with potency commensurate with their high drug load (Hamblett et al., 2004; Adem et al., 2014). However, ionic or thermal stress revealed a disproportionate tendency for the high DAR species to aggregate and fragment (Adem et al., 2014). Importantly, potency in vivo was severely compromised by rapid clearance of the DAR8 ADC, whereas DAR4 appeared more optimal (Hamblett et al., 2004). The implied relationship between DAR, clearance, and efficacy is illustrated in Fig. 3B.

B. Uniform Site-specific Conjugation

To circumvent some of the issues associated with nonuniform drug loading, site-directed conjugation was developed. The prototypical version involved substitution of a specified amino acid in the IgG1 heavy or light chain with a cysteine, resulting in two exclusive sites of conjugation per antibody tetramer (Junutula et al., 2008). The resulting THIOMAB-MMAE conjugate surprisingly exhibited efficacy comparable to a stochastically drugged ADC containing nearly twice as much MMAE after dosing of equimolar amounts of antibody protein. Improvements in safety were also noted, culminating in an enhanced apparent therapeutic index. A comparison of THIOMABs containing MMAE appended to distinct sites, revealed a pronounced impact of conjugation site on linker-drug stability, which in turn, dramatically impacted efficacy (Shen et al., 2012). Thus proper conjugation site selection offers an alternative approach to chemical modification as a means to achieve enhanced stability. Cysteines may also be engineered into the carboxy- or amino-termini of IgG polypeptides where they can be reacted with drugs derivatized with a sulfhydryl or aldehyde group (Bernardes et al., 2013).

Several alternatives to the THIOMAB technology, facilitating conjugation of drugs at specific sites, have recently emerged (reviewed by Panowski et al., 2014). One of them involves the use of the amber suppressor tRNA/aminoacyl-tRNA synthetase pair that enables coded translation of an unnatural amino acid into a specified position in the antibody. Incorporation of *p*-acetylphenylalanine provides a keto group as a reactive site for formation of a stable oxime with an alkoxyamine derivatized drug (Axup et al., 2012). Tested as an anti-HER2-*p*-acetylphenylalanine-auristatin conjugate, the ADC inhibited xenograft tumor growth. The clearance and exposure parameters of the ADC were very similar to the corresponding naked antibody. An additional embodiment in which an orthogonal reactive group is introduced involves the incorporation of a

selenocysteine amino acid at the C terminus of the IgG polypeptide chain (Hofer et al., 2009). This was accomplished by engineering a 3' TGA codon along with a proximal selenocysteine insertion sequence element derived from the thioredoxin reductase 1 cDNA. The TGA then codes for incorporation of the naturally occurring amino acid selenocysteine at the C terminus of the modified IgG chain. The seleno group offers a unique site on the antibody for electrophilic attack, resulting in covalent conjugation of appropriately derivatized compounds.

Additional methods for site-specific conjugation include aldehyde tagging, wherein a short consensus sequence, CXPXR, is engineered into eight different regions of the IgG heavy and light chain polypeptides (Drake et al., 2014). The sequence is recognized by formyl glycine generating enzyme, resulting in the conversion of the consensus cysteine to a formyl glycine containing a reactive aldehyde group. Hydrazino-iso-Pictet-Spengler chemistry was then used to generate a stable bond to the reactive aldehyde group. An example conjugate containing maytansine appended to anti-HER2 was found to be stable and efficacious in vivo. Three independent insertion sites were evaluated, and similar to the THIOMAB, the site of conjugation impacted the stability and corresponding efficacy of the ADCs. In another approach using a transposable consensus sequence, Strop et al. (2013) exploited microbial transglutaminase (mTG), which catalyzes cross-linking of glutamine side chains to primary amines. Hence, a primary amine acyl acceptor can be covalently coupled to the glutamine in the presence of mTG. The glutamine tag, LLQG, was inserted into a variety of positions in the IgG chains, and a number of highly reactive sites were identified. One of the reactants tested was acyl-lysine-monomethyl dolastatin 10 (MMAD), resulting in a potent ADC with an approximate DAR of two. Preclinical efficacy of an acyl-lysine-MMAD ADC targeting the antigen M1S1 was impressive and comparable to that of a stochastically drugged ADC containing nearly twice as much MMAD. Site-dependent differences in pharmacokinetics, particularly in rat, were again noted.

Dennler et al. (2014) demonstrated an additional method involving mTG catalyzed crosslinking, but lacking the requirement for any engineering of the antibody cDNA sequence. The approach takes advantage of conserved heavy chain residue glutamine-295, which is the only site recognized by mTG in deglycosylated IgG. Although direct conjugation of auristatins prederivatized with linker containing the amine donor was demonstrated, the yields were poor and required a 40-fold excess of linker-drug per GLN-295 site. Better yields were realized, at much lower linker-drug to GLN-295 site ratios, when bifunctional linkers containing the amine donor at one end, and either an S-protected thiol or azide at the other, were first coupled to GLN-295.

This was followed by reacting the appropriately derivatized auristatin linker-drug construct with the pre-existing GLN-295 adduct. One potential liability is the lack of carbohydrate on the antibody, although it remains unclear whether this would affect the pharmacokinetics or activity of an ADC in humans.

Direct chemical modification of antibody carbohydrate to a site reactive for conjugation was described by Zuberbuhler et al. (2012). Here, sodium periodate was used to selectively oxidize the fucose moiety in the N-linked glycan to an aldehyde, thereby making it reactive with a hydrazide derivative of a dolastatin. The resulting hydrazone trigger in the ADC is not particularly stable, though, exhibiting a half-life of about 18 hours at physiologic pH in phosphate-buffered saline. Carbohydrate as a site of antibody conjugation was also described by Okeley et al. (2013), but in this scheme the reactive site was generated by metabolic incorporation of derivatized fucose into the N-linked glycan. The antibody is produced by Chinese hamster ovary cells in media containing 1 mM of a fucose analog substituting for native fucose as a substrate for fucosyltransferase VIII. Thus, incorporation of 6-thiofucose into the glycan presented a unique free thiol that facilitated conjugation by Michael addition with a maleimide containing linker drug. The ADC drugged with MMAE in this fashion was quite stable, losing only 15% of its drug over 4 days in plasma. The average DAR was only 1.3, largely owing to incomplete incorporation of the fucose analog into the glycan.

V. Targets

Assessing both the relative expression level of a target on tumor and normal tissues and its representation across a large panel of tumors is a critical first step in selecting a target for ADC therapy. This was facilitated markedly by the advent of high-throughput technologies enabling the measurement of thousands of mRNA transcripts across thousands of tissue samples. One could readily identify putative cell surface proteins overexpressed in a significant portion of tumors while lacking expression in particularly vital or regenerative normal tissues. Subsequent validation steps included verifying the abundance and cell surface localization of the target protein as well as its ability to internalize a bound antibody. However, the paradigmatic target—highly expressed on cancer cells and altogether absent on normal cells—is extremely rare, if not nonexistent. As some clinical failures attest, the target does matter, both for efficacy and safety. It is far too expansive to cover all targets entertained for ADC therapy, but some select examples can help convey lessons learned from the clinical experience.

A. Human Epidermal Growth Factor Receptor II—Is Level of Expression Important?

It is tempting to trumpet the success of Kadcyla (Trastuzumab-SMCC-DM1) as a harbinger of future triumphs of ADCs in solid cancers. However, with its multimillion copies of oncogenic receptor per tumor cell driven by DNA amplification and a drug conjugated to an already functionally neutralizing antibody, HER2 is hardly a representative ADC target (Kallioniemi et al., 1992; Burris et al., 2011b). Therefore it is difficult to gauge the general applicability of the SMCC-DM1 linker-drug used in Kadcyla based solely on the HER2 experience. The SMCC linker yields a released maytansinoid drug with little bystander cell killing (Erickson et al., 2010), yet performs quite well in the context of Kadcyla. One could conclude that the exceptionally high and relatively homogenous target expression of HER2 may enable use of the SMCC linker. However, the results of additional clinical investigations with SMCC-DM1 ADCs, such as AMG 172 and AMG 595, targeting CD70 and EGFR (Table 1), respectively, should help determine whether this linker-drug configuration has broad utility. We have learned from Kadcyla that the level of target expression may correlate with outcome. For patients with tumors diagnostically HER2 positive by fluorescent in situ hybridization, the overall response rate was 40% compared with 20% for those scored as HER2 normal (Krop et al., 2012). In this study, an exploratory analysis in which only HER2-positive tumors were further ranked by reverse-transcription polymerase chain reaction or fluorescent in situ hybridization revealed a trend for improved outcome for patients whose tumor biopsy samples scored above the median. Additional positive correlations between HER2 mRNA transcript levels, as measured by reverse-transcription polymerase chain reaction, and patient outcomes have been reported in other studies (Burris et al., 2011a; Perez et al., 2014).

B. CD30—Does Antibody Effector Function Contribute to Efficacy?

To what extent does the recruitment of effector cells by the antibody portion of the ADC contribute to its activity? To address this, Adcetris (brentuximab vedotin), which targets CD30 and produces excellent response rates in refractory Hodgkin's lymphoma (Younes, 2014), provides an example less encumbered than Kadcyla, because unlike HER2, it is not expressed at unusually high levels nor is it oncogenic. It is a member of the tumor necrosis factor receptor family, and although its function remains unclear, a variety of mouse models implicate it in the regulation of autoimmune responses. Normal tissue expression of CD30 is primarily restricted to activated immune cells, whereas positive lymphoid cancers express considerably higher

levels. Also dissimilar to HER2, naked antibodies to CD30, including SGN-30 used in Adcetris, have not generated compelling responses in the clinic, particularly in Hodgkin's lymphoma (reviewed by Kumar and Younes, 2014). Moreover, a variety of clinical investigations were initiated, including radioactive and proteinaceous toxins appended to anti-CD30, as well as bispecific antibodies, but none have progressed beyond early stage clinical testing (Kumar and Younes, 2014). Together, these clinical failures, especially that of SGN-30, contrasted with the remarkable success of Adcetris, suggest that anti-CD30 itself contributes little in the way of effector function but rather acts primarily as a means for specifying the delivery of MMAE. Nevertheless, one could credit some of the success of Adcetris to its application in hematologic cancers, where approved antibody therapeutics are disproportionately represented (Niwa and Satoh, 2015). Numerous disparate clinical ADC trials (Table 1), wherein the same VC-MMAE linker-drug configuration in Adcetris is aimed at other targets, will help define the specific attributes that contribute to success.

C. CD79 versus CD22—How Important Is the Specific Target?

It also remains possible that CD30 is simply a spectacular target for ADC therapy. Although we have learned from clinical correlations with Kadcyra that high target expression is beneficial, the rules that determine the utility of a potential ADC target remain murky. The degree to which the specific target per se influences safety and efficacy of an ADC can be glimpsed from a recent Phase II trial in which antibodies to CD22 and CD79b, both conjugated to VC-MMAE, were tested side by side in relapsed/refractory non-Hodgkin's lymphoma (Morschhauser et al., 2014). CD79b is a positive signaling component of the B-cell receptor, whereas CD22 is a B-cell specific transmembrane glycoprotein that negatively regulates antigen receptor signaling (Chu and Arber, 2001; Sullivan-Chang et al., 2013). Both targets are detected on normal adult B-cells and amply expressed on the vast majority of NHL. The two corresponding ADCs equipped with VC-MMAE produced similar findings in preclinical efficacy and safety studies, including the target-dependent killing of normal B-cells in nonhuman primates (Dornan et al., 2009; Li et al., 2013). In the Phase II trial, the two ADCs, combined with Rituxan jointly by Biogen-Idec (Cambridge, Mass) and Genentech (South San Francisco, CA), were well-tolerated with similar primary toxicities, consisting of neutropenia, peripheral neuropathy, and diarrhea, and very comparable overall response rates in diffuse large B-cell lymphoma—22/39 (6 CR+16 PR) and 24/42 (10 CR+14 PR) for CD79b and CD22, respectively. So, despite their different structures and opposing functions, the two targets are nearly indistinguishable with respect to MMAE ADC therapy. This is not to say that

target selection is irrelevant—CD22 and CD79b satisfied a battery of preclinical qualifications, including efficacy and safety, before consideration for further development in the first place. But, having qualified, they seem equally competent despite their differing functional and biochemical backgrounds.

D. gpNMB and CD44—Is There Dose Limiting Target-dependent Toxicity?

These two targets have very little in common biologically, structurally or otherwise, and the clinical studies targeting them with ADCs used different antimetabolic drugs. Nevertheless, they share a common side effect not common to other ADCs containing their respective antimetabolic drugs. Glycoprotein nonmetastatic melanoma protein B (gpNMB), the melanoma-related glycoprotein homolog of the pigment forming protein premelanosome protein 17 (PMEL-17), is expressed on the surface of epidermal melanocytes *in situ* (Tomihari et al., 2009; Naumovski and Junutula, 2010). Glematimumab vedotin (anti-gpNMB-VC-MMAE) has been investigated in advanced melanoma, where skin rash was identified as the most common adverse event (Ott et al., 2014). At the MTD of 1.88 mg/kg administered every 3 weeks, 30% of patients experienced grade 3 or higher skin rash. Nevertheless, partial responses were observed in 13% (5/40) of this cohort, and the MTD, although attained for different reasons, is similar to that reached for the approved VC-MMAE ADC Adcetris (Younes et al., 2012b). A separate study involving anti-CD44v6 conjugated to DM1 also evoked skin toxicities, again not typically observed with maytansinoid conjugates. In this Phase I study of bivatuzumab mertansine, 24/31 patients experienced dose-dependent skin events involving rash, blister formation, skin desquamation, which included a fatal case of toxic epidermolysis, ultimately prompting discontinuation of the investigation. The expression of CD44v6 on normal keratinocytes likely accounted for the skin toxicities. It seems that the lesson learned from these two ADC studies is to avoid targets expressed in normal skin tissue.

E. NaPi2b and Mesothelin—Will Expression on Normal Tissue Always Result in Target-dependent Toxicities?

Although it is clear that high normal tissue expression can present a liability, it is not universally the case. The target sodium phosphate transporter 2b (NaPi2b), a sodium phosphate transporter and product of the SLC34A2 gene, is well expressed in normal lung tissue and is clearly critical for normal function there, as evidenced by the linkage of SLC34A2 germ line mutations to heritable pulmonary alveolar microlithiasis (Traebert et al., 1999; Corut et al., 2006). Yet in a Phase I study in which anti-NaPi2b-VC-MMAE was administered to 30 ovarian cancer patients, including 18 treated

at 1.8–2.8 mg/kg, pulmonary toxicity was not reported (Gordon et al., 2013). Most toxicities were constitutional, whereas those resulting in dose limitations, such as peripheral neuropathy, were generally consistent with VC-MMAE ADCs aimed at orthogonal targets. Thus the propensity for target-dependent toxicity may depend on the particular normal tissue expressing the target. The reasons for this remain unclear, but could relate to the selectivity of antimitotic drugs, including MMAE and DM1, for proliferating or regenerative tissues, such as skin and bone marrow. By contrast, lung tissue, in the absence of injury, is not highly regenerative. Secondly, NaPi2b expression is confined to the apical surface of large cuboidal type II pneumocytes (Traebert et al., 1999), which could potentially hinder the access of the ADC to the target in normal tissue.

Mesothelin (MSLN), a 40-kDa glycoposphatidylinositol-anchored protein, is expressed normally on the surface of mesothelial cells lining the pleura, peritoneum, and pericardium and is overexpressed on a variety of solid tumors (Hassan and Ho, 2008). An ADC targeting MSLN and armed with VC-MMAE was evaluated in a Phase I clinical trial for the treatment of pancreatic cancer (Weekes et al., 2014). Some grade 3/4 toxicities, including neutropenia, AST/ALT elevation, and fatigue were reported but were not consistent with disruption of mesothelial linings. By contrast, the targeting of MSLN with the recombinant immunotoxin SS1P [SS1(dsFv) PE38] provoked a dose-limiting toxicity of grade 3 pleuritis, characterized by fever, hypoxia, pleural effusion, and pain (Hassan et al., 2007). Grade 1/2 pericardial effusion was also noted. These are quite likely target-dependent events but were not observed with anti-MSLN-VC-MMAE. A critical distinction here is the payload in SS1P, *Pseudomonas* exotoxin A, which kills cells by inhibiting protein synthesis, as opposed to the antimitotic MMAE. This implies that target-dependent toxicity might also depend on the mechanism of action of the cytotoxin. Additionally, SS1P is somewhat smaller than an ADC, consisting of an IgG Fv fragment fused to a 38-kDa portion of the *Pseudomonas* exotoxin A, which could facilitate better access to the mesothelium.

F. Eph Family Receptor Tyrosine Kinase A2—Is Target-dependent Toxicity Predictable?

EphA2 is an Eph family receptor tyrosine kinase expressed in a variety of cancers that was recently targeted with MEDI-547, an anti-ephA2 antibody conjugated to MMAF using the noncleavable MC linker (Annunziata et al., 2013). The clinical experience here helps exemplify the difficulties of target evaluation. Clinical studies evaluating SGN-75 and SGN-CD19a, both of which also contain MC-MMAF but target CD70 and CD19, respectively, provide a background for evaluating adverse events driven specifically by the

antibody portion of MEDI-547 (Borate et al., 2013; Tannir et al., 2014). In striking contrast to SGN-75 and SGN-CD19a, which were tolerated at doses as high as 3 and 6 mg/kg, respectively, MEDI-547 was discontinued because of drug-related adverse events after dosing the first cohort at 0.08 mg/kg (Annunziata et al., 2013). Bleeding and coagulation events reported for five of the six patients, three of which were recorded as severe adverse events, were responsible for termination of the study. These pathologies were not entirely surprising because they were observed preclinically in cross-reactive rats and primates, albeit at much higher doses than that attained in humans. The toxicities are probably target dependent, but the specific tissue(s) involved in eliciting the adverse events were not identified. Although GLP compliant tissue cross reactivity studies were performed, this practice involves the immunohistochemical application of the drug itself, in this case MEDI-547, to frozen tissue sections as a means of detecting potential sites of reactivity (Leach et al., 2010). Accordingly, some weak staining restricted to tonsillar and esophageal epithelium was noted for MEDI-547. However, a more rigorous analysis using an antibody exhibiting demonstrable attributes as an immunohistological reagent would be more compelling. The authors concluded that a reassessment of tissue cross reactivity was warranted. It also remains possible that the specific antibody per se, either on or off target, was the driver of toxicity.

G. MUC1 and MUC16—What are Consequences of Shed Target Antigen?

These two examples address the consequences of circulating shed target antigen on the performance of the ADC. Muc16 is an extremely large cell surface antigen containing multiple tandem mucin domains, which bind the antibody in DMUC5754A, an ADC containing the VC-MMAE linker-drug (Chen et al., 2007). Preclinically, two distinct antibodies were tested as ADCs against Muc16-11D10, which bound a non-repeating epitope, and 3A5, which recognized multiple mucin repeats. Despite having lower average affinity, the 3A5 ADC was vastly superior to that of 11D10, supporting the notion that higher antibody target density correlates with greater efficacy. However, the presence of these multiple mucin repeats on CA125—the extracellular portion of Muc16 that is shed into circulation—evoked fear of toxic ADC immune complexes as well as pharmacokinetic interference. None of this came to bear in the clinic, though, because there was no impact on efficacy, safety, or pharmacokinetic parameters that could be attributed to CA125 levels, ranging from very low (100 U/ml) to very high (7177 U/ml), in patients administered the recommended Phase II dose of 2.4 mg/kg (Liu et al., 2013).

Cantuzumab mertansine, an early iteration of a maytansinoid ADC targeting Muc1, or CanAg, was

investigated in cancer patients ranging widely in plasma levels of shed CanAg (Tolcher et al., 2003). Of the 30 patients with detectable plasma CanAg levels, 25 experienced rapid reductions to undetectable levels after the first dose of the ADC, which was maintained for up to 21 days in the majority of the patients. Again, shed antigen levels did not impact pharmacokinetics nor exhibit any relation to toxicity. However, an impact of shed CanAg was called out in a Phase II study of huC242-DM4, a subsequent iteration of a maytasinoid ADC targeting Muc1 (Goff et al., 2009). In this study, exposure of huC242-DM4 in patients with gastric cancer was inversely correlated to plasma CanAg levels. Interestingly, those with low levels of CanAg, and thus higher circulating levels of huC242-DM4, appeared more susceptible to the ocular toxicity associated with this class of ADC than those with high CanAg. Accordingly, the study was amended to administer a higher dose of huC242-DM4 to patients with high plasma CanAg. Although it was apparent from this study that shed antigen was likely binding to and promoting the clearance of ADC, the presumed formation of ADC-target immune complexes did not exacerbate toxicity.

VI. Conclusions and Perspectives

The expanding register of ADCs under clinical investigation certainly attests to the interests and hopes we harbor for ADC therapeutics in the oncology clinic. However, there have been a fair number of failures, many of which have occurred quite recently. Although

some were grounded by insufficient activity, it is apparent that even a marginal gain in tolerability could have prevented their failure. Such untoward safety signals can arise from at least four potential sources (Fig. 4). Normal cells expressing target are a proven liability, although not a common one, so far. Systemic deconjugation of ADCs certainly occurs and can be monitored, but the consequences for safety depend on the nature and amount of released drug or drug adduct. Nevertheless, toxicity abounds even when highly stable linkers are used or when the released free drug is relatively impotent.

On balance, toxicity unrelated to target expression is probably the most significant obstacle hampering the progression of ADCs. The catabolism of ADCs resulting from their pharmacological clearance yields intracellular amounts of the very drug intended to kill the cancer cell. Furthermore, once liberated, the drug can diffuse into neighboring tissues or escape into circulation and, depending on its nature, kill additional cells through the bystander effect. Even the successes, of which formally there are just two, would provide better benefit if not for this target-independent toxicity. HER2 is not expressed on megakaryocytes nor is CD30 on peripheral neurons, yet therein lie the reasons for dose limitations and reductions of Kadcyla and Adcetris, respectively. Thus it is likely that these toxicities emanate, either directly or indirectly, from uptake and conversion of the prodrug, i.e., the ADC, to the active small molecule drug by cells lacking target. This underscores the need to exploit the differences between nonspecific, pinocytotic uptake and target driven uptake of the ADC, as well any differences by which normal and cancer cells process the prodrug to drug.

Altering the antibody in ways that reduce either the amount or rate of uptake by cells lacking target might enhance the safety margin of ADCs. Obviously, appending drugs that exhibit greater selectivity toward cancer cells would also be beneficial. In principal, highly potent "targeted" therapies that exploit clearly defined genetic differences in cancer cells could be made more effective and safer if conjugated to antibodies. Similarly, drugs designed to take advantage of more generalized attributes of cancer that distinguish them from normal cells, such as hypoxia or endoplasmic reticulum (ER) stress, could benefit from conjugation to antibodies. Although such drugs are designed to be more selective to cancer, conjugating them to antibodies could further reduce toxicities, improve pharmacokinetic exposure, and facilitate entry of impermeable molecules into targeted cells. Some of these efforts, and other potential improvements, are underway.

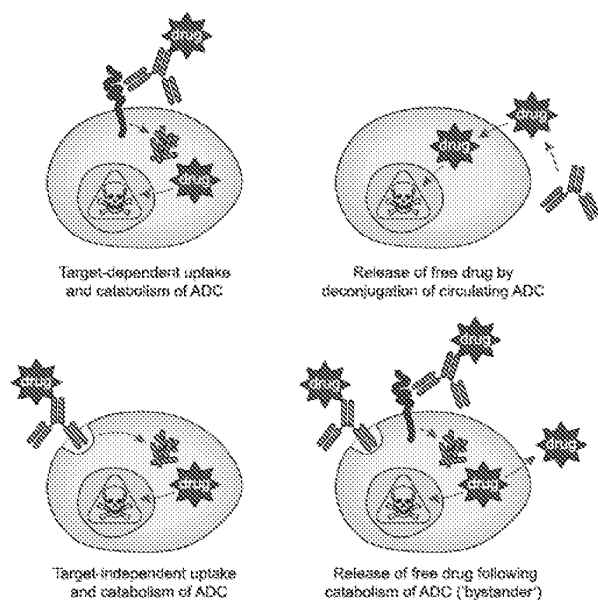


Fig. 4. Possible mechanisms of ADC toxicity. Normal cells expressing target uptake the ADC (upper left); free drug released from ADC in circulation diffuses into normal cells (upper right); nonspecific uptake of ADC by normal cells (lower left); free drug released from cells that uptake ADC, specifically or nonspecifically, diffuses into normal cells.

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Antibody–drug conjugates as novel anti-cancer chemotherapeutics

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Synopsis

Over the past couple of decades, antibody–drug conjugates (ADCs) have revolutionized the field of cancer chemotherapy. Unlike conventional treatments that damage healthy tissues upon dose escalation, ADCs utilize monoclonal antibodies (mAbs) to specifically bind tumour-associated target antigens and deliver a highly potent cytotoxic agent. The synergistic combination of mAbs conjugated to small-molecule chemotherapeutics, via a stable linker, has given rise to an extremely efficacious class of anti-cancer drugs with an already large and rapidly growing clinical pipeline. The primary objective of this paper is to review current knowledge and latest developments in the field of ADCs. Upon intravenous administration, ADCs bind to their target antigens and are internalized through receptor-mediated endocytosis. This facilitates the subsequent release of the cytotoxin, which eventually leads to apoptotic cell death of the cancer cell. The three components of ADCs (mAb, linker and cytotoxin) affect the efficacy and toxicity of the conjugate. Optimizing each one, while enhancing the functionality of the ADC as a whole, has been one of the major considerations of ADC design and development. In addition to these, the choice of clinically relevant targets and the position and number of linkages have also been the key determinants of ADC efficacy. The only marketed ADCs, brentuximab vedotin and trastuzumab emtansine (T-DM1), have demonstrated their use against both haematological and solid malignancies respectively. The success of future ADCs relies on improving target selection, increasing cytotoxin potency, developing innovative linkers and overcoming drug resistance. As more research is conducted to tackle these issues, ADCs are likely to become part of the future of targeted cancer therapeutics.

Key words: antibody–drug conjugates, brentuximab vedotin, cancer, chemotherapy, monoclonal antibodies, trastuzumab emtansine.

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INTRODUCTION

The advent of modern-day cancer chemotherapy dates back to the mid-1900s when a chemical warfare agent known as nitrogen mustard was seen to destroy the bone marrow and lymph tissue of exposed individuals [1]. In the following years, nitrogen mustard, along with numerous other alkylating agents [2] took centre stage in the treatment of various haematological malignancies including leukaemia, lymphoma, Hodgkin's disease and multiple myeloma. Several other serendipitous observations [3] lead to the development of the first primitive classes of cytotoxins (Figure 1). Despite vast progress in the field of cancer chemotherapy, small-molecule cancer drugs (although highly potent)

continue to be plagued with the problems of non-specific toxicity (as a result of targeting all rapidly dividing cells), narrow therapeutic windows [4] and increasing resistance rates [5]. These concerns emphasize the need to move away from conventional cancer treatments and explore new ways to tackle the ever-present disease.

In recent years, enhanced understanding of cancer biology has shifted the focus of cancer treatment from traditional chemotherapy to targeted cancer therapies that take advantage of the differentiating features of tumour cells to provide a framework for drug development. These distinctive features, collectively known as the 'hallmarks of cancer' [7,8] enable tumour cells to survive, multiply and metastasize using a variety of mechanisms including activation of self-sufficient growth signals, evasion of

Abbreviations: ADC, antibody–drug conjugates; ADCC, antibody-dependent cellular cytotoxicity; AML, acute myeloid leukaemia; CD, cluster designation; CDC, complement-dependent cytotoxicity; CDCC, complement-dependent cell-mediated cytotoxicity; CEA, carcinoembryonic antigen; CH, constant heavy chain; DAR, drug–antibody ratio; DM, maytansinoid; EGFR, epidermal growth factor receptor; FcRn, neonatal Fc receptor; FDA, Food and Drug Administration; HER2, human epidermal growth factor receptor 2; mAb, monoclonal antibody; MAPK, mitogen-activated protein kinase; MMAE/E, monomethyl auristatin E/F; MRP, multi-drug resistance protein; NK, natural killer; P-gp, P-glycoprotein; PBD, pyrotetrazodiazepine; PI3K, phosphoinositide 3 kinase; PSMA, prostate-specific membrane antigen; T-DM1, trastuzumab emtansine.

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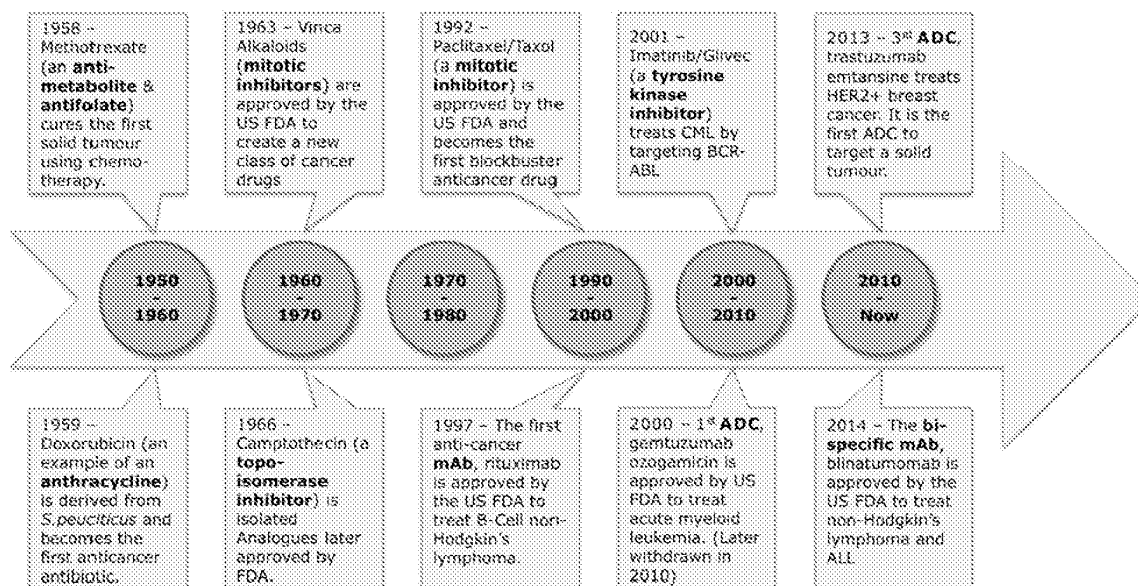


Figure 2 Evolution of chemotherapeutic drugs [6]

anti-growth signals, evasion of apoptosis and induction of angiogenesis. Currently approved targeted therapies counteract these and provide safer and more efficacious alternatives to traditional chemotherapy.

Cancer cells differ from normal cells due to genomic mutations in oncogenes and/or tumour suppressor genes [9]. Once the integrity of the genome is compromised, cells are more likely to develop additional genetic faults, some of which may give rise to tumour-specific antigens (found only on the surface of tumour cells) or tumour-associated antigens (overexpressed on tumour cells, but also present on normal cells) [10]. Ongoing research has found that several human cancers express unique tumour-specific or tumour-associated cell surface antigens [11] which are of great value as targets for large molecule, monoclonal antibody (mAb)-based therapy.

The use of antibodies as 'magic bullets' to treat disease was first proposed more than 100 years ago by the founder of chemotherapy, Paul Ehrlich [12]. Due to several challenges in the development of human antibodies, it was only in 1997 that the US FDA (Food and Drug Administration) approved the first anti-cancer antibody, rituximab, for the treatment of B-cell non-Hodgkin's lymphoma [13]. Early mAbs were based on murine or chimeric antibodies that were modified to target human antigens. As these were non-human antibodies, they evoked a strong immune response that prevented the treatment from being successful. The large size of the mAbs also proved to be problematic as it resulted in reduced tumour penetration [14] and poor therapeutic effect. Since then, several advances in antibody engineering [15,16] have optimized pharmacokinetics and effector function while reducing immunogenicity. This has resulted in a significant increase

in the development of antibody-based drugs [17,18] against cancer.

mAbs exert their therapeutic effect [19] by binding to tumour-specific or tumour-associated cell surface antigens. Once bound, the mAb kills the tumour cell by one or more of the following mechanisms; (i) abrogation of tumour cell signalling, resulting in apoptosis (ii) modulation of T-cell function through antibody-dependent cellular cytotoxicity (ADCC), complement-dependent cytotoxicity (CDC) or complement-dependent cell-mediated cytotoxicity (CDCC) and (iii) exertion of inhibitory effects on tumour vasculature and stroma [20,21]. Despite these various cell-killing mechanisms, most mAbs display insufficient cytotoxic activity [22]. Current efforts in cancer treatment have therefore focused on combining the selectivity of mAbs with the potency of chemotherapeutic small molecules, giving rise to an entirely new class of anti-cancer drugs known as antibody-drug conjugates (ADCs) [23].

ADCs are tripartite drugs comprising a tumour-specific mAb conjugated to a potent cytotoxin via a stable linker (Figure 2). The three components of the ADC together give rise to a powerful oncolytic agent capable of delivering normally intolerable cytotoxins directly to cancer cells, which then internalize and release the cell-destroying drugs [24]. Although the concept of combining a mAb with a cytotoxic drug is fairly old, significant improvements have been made since the first generation of ADCs. Early ADCs (based on mAbs that did not undergo internalization) were engineered to release their active drug once tumour-specific enzymes (such as matrix metalloproteinases) or tumour-specific environments (such as a lower pH) cleaved the linker component of the ADC [25]. These non-internalizing ADCs failed to significantly

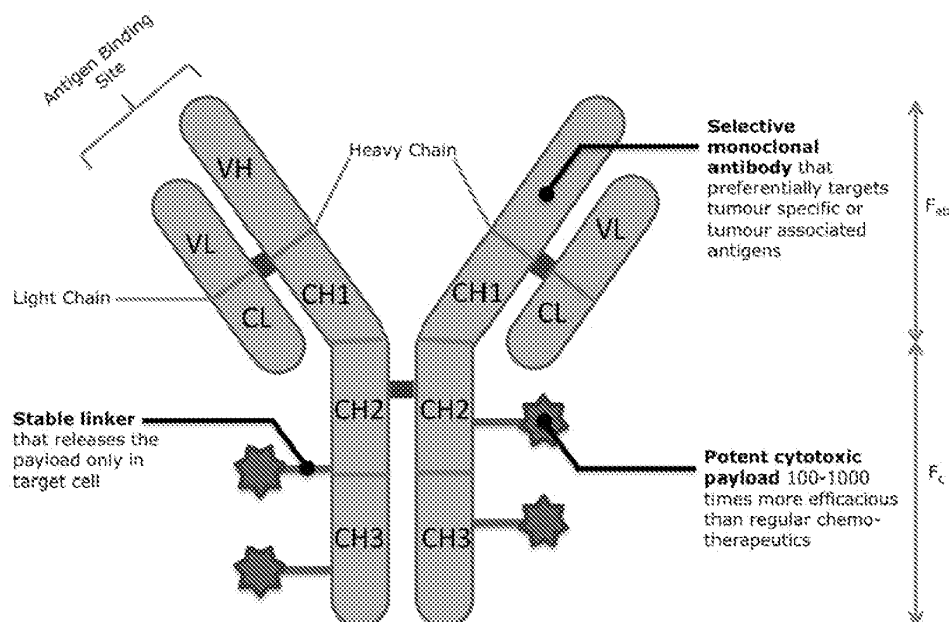


Figure 2 Structure of an ADC [28]

improve drug specificity and therefore did little to decrease toxicity [26]. Since then, the development of internalizing conjugates has increased their therapeutic potential. The targeted nature of ADCs allows for increased drug potency coupled with limited systemic exposure. Together, these features clinically manifest themselves as fewer side effects and a wider therapeutic window. In addition, the internalization of the ADC reduces drug resistance arising from P-glycoprotein (P-gp)-mediated efflux mechanisms [27]. ADCs are therefore capable of bypassing major issues in both traditional and targeted chemotherapy.

Although the concept of ADCs is theoretically simple, it is difficult to combine its various components into an optimized and functional therapeutic agent. To date, three ADCs have gained entry into the market, of which only two remain [29]. The first ADC to obtain FDA approval was gemtuzumab ozogamicin (Mylotarg®), marketed by Wyeth (now Pfizer), for the treatment of relapsed acute myeloid leukaemia (AML) [30]. In 2010, a decade after its approval, gemtuzumab ozogamicin was withdrawn from the market when a clinical trial showed that it had little benefit over conventional cancer therapies [31] and was associated with serious hepatotoxicity [32]. This could have been due to the fact that the linker technology used was not stable enough to prevent the drug from being released in the bloodstream [33]. The two ADCs currently in the market are brentuximab vedotin (Adcetris® by Seattle Genetics) and trastuzumab emtansine (T-DM1; Kadcyla® by Genentech). The former is the first of two ADCs to be approved and is currently being used to treat patients with relapsed or refractory Hodgkin's lymphoma or those with

relapsed or refractory systemic anaplastic large cell lymphoma [34]. The more recent approval of T-DM1, in 2014, [35] for use against breast cancer, proved that ADCs were capable of targeting solid tumours in addition to haematological malignancies. Although there are only 2 ADCs currently in the market, there are more than 30 ADCs being developed to target a wide range of blood cancers and solid tumours. In contrast with small molecules that have a limited choice of drug targets, the diverse range of ADC targets results in a robust drug pipeline with minimal overlap between different pharmaceutical companies [36]. With the recent FDA approvals of brentuximab vedotin and T-DM1, there has been an increase in research investigating the use of ADCs in the treatment of cancer. Recent reviews on ADCs have summarized the preclinical and clinical advances made in the field and have discussed key characteristics of marketed ADCs [37–39]. They have also provided an overview of linker properties, drug releasing strategies and viable targets for the design of ADCs [40,41]. The primary objective of this article is to produce an updated, comprehensive review of the current knowledge in these areas, based on the developments in the last few years.

MECHANISM OF ACTION OF ADCs

When designing an ideal ADC, it is essential to understand the mechanism of action in order to identify the desired features of

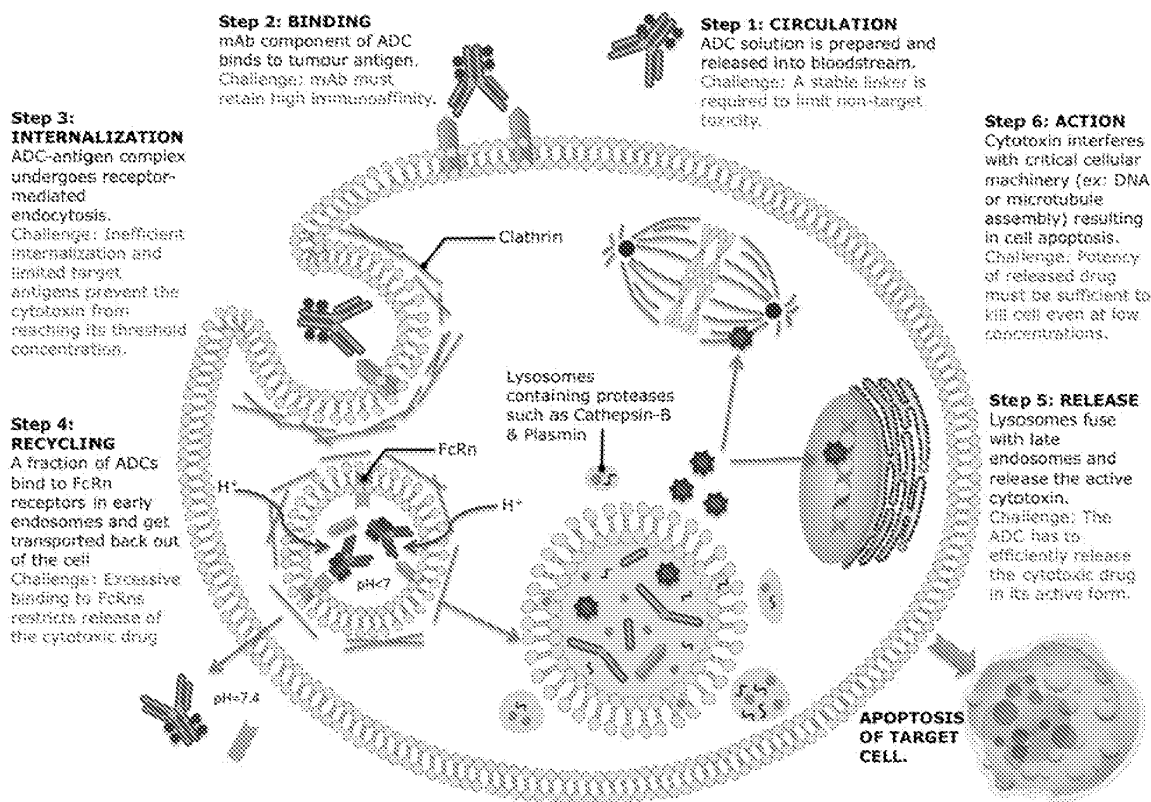


Figure 3 Mechanism of action of ADCs

each of its three components. An ideal ADC is one that retains the selectivity and killing capacity of a mAb while still being able to release the cytotoxic drug in quantities large enough to kill tumour cells. Each of the steps involved in the mechanism of action is associated with unique challenges [33] that complicate the design of ADCs. These are illustrated in Figure 3.

ADCs are administered intravenously in order to prevent the mAb from being destroyed by gastric acids and proteolytic enzymes. The mAb component of the ADC enables it to circulate in the bloodstream until it finds and binds to tumour-specific (or tumour-associated) cell surface antigens present on target cancer cells. In the interest of preventing unwarranted release of the cytotoxin and maximizing drug delivery to cancer cells, an ideal linker would not only have to be stable in the bloodstream but also capable of releasing the active form of the cytotoxic drug when required [42].

Once the mAb component of the ADC is bound to its target antigen, the ADC-antigen complex should theoretically be internalized via receptor-mediated endocytosis. The internalization process finishes with the formation of a clathrin-coated early endosome containing the ADC-antigen complex [43]. An influx of H^+ ions into the endosome results in an acidic environment that facilitates the interaction between the mAb component of a

fraction of ADCs and human neonatal Fc receptors (FcRns). The bound ADCs are transported outside the cell, where the physiological pH of 7.4 enables the release of the ADC from the FcRn [44]. This mechanism acts as a buffer for preventing the death of healthy cells in the case of ADC mis-delivery. Excessive binding of ADCs to tumour cell FcRns might however restrict the release of the cytotoxic drug and prevent the ADC from taking effect [45]. FcRn expression is primarily within the endosomes of endothelial cells.

ADCs that remain in the endosome without binding to FcRn receptors form the late endosome. These subsequently undergo lysosomal degradation, allowing the release of the cytotoxic drug into the cytoplasm. At this stage, it is crucial to ensure that a sufficient (i.e. threshold) concentration of the drug is present within the cancer cell for its destruction to be guaranteed. This is however complicated in practice by the facts that cell-surface antigens are often quite limited and the process of internalization, rather inefficient [46]. Assuming all the steps involved in the mechanism of ADC action have an efficiency of 50%, only 1%–2% of the administered drug will reach tumour cells [47]. This makes the choice of cytotoxin particularly important, as it is required to be highly efficacious at very low concentrations. Drugs that are usually unsuitable for normal chemotherapy (due

to excessive toxicity) are therefore a necessary component of ADCs.

Different classes of cytotoxic drugs result in cell death using various mechanisms [39]. The common element between all the classes is interference with critical cell functioning and, as a consequence, either direct killing of the cell or induction of apoptosis. As targeted cancer cells die, there is potential for the active cytotoxic drug to kill neighbouring tumour cells and the supporting stromal tissue. The design of ADCs with respect to the choice of target, mAb, linker and cytotoxin are all very important determinants of whether or not the threshold concentration of the cytotoxic drug is reached within the tumour cell. These factors therefore determine the overall success of an ADC.

Target Antigen Selection

Successful development of an ADC is dependent on the selection of an appropriate target antigen to which the mAb component of the ADC can bind. Aside from being tumour-specific or tumour-associated, cell surface antigens should also undergo efficient internalization, have high levels of expression and possess high penetrance, a characteristic whereby a large percentage of tumour samples test positive for the presence of the antigen. The target of gemtuzumab ozogamicin, an ADC previously used against AML, was cluster designation 33 (CD33), a transmembrane cell-surface glycoprotein expressed on the surface of mature and immature myeloid cells. CD33 has extremely high penetrance with 90%–95% of all AML patients testing positive for the antigen [48]. With regard to tumour specificity and sensitivity however, CD33 performed rather poorly as it was found to have only low levels of expression on not only on mature and immature myeloid cells but also erythroid cells, megakaryocytes and multipotent progenitor cells [49,50]. Current ADCs aim to execute their therapeutic action by identifying target antigens that fulfil all four of their requirements.

Although tumour-specific antigens are ideal, most ADC targets tend to be tumour-associated. The expression of such antigens should be kept to a minimum on healthy tissue cells, [51–53] unless these cells are insensitive to drug action. Examples of target antigens that are expressed in both normal tissues as well as cancer tissues include the prostate-specific membrane antigen (PSMA) and the HER2 (human epidermal growth factor receptor 2) receptor [38]. In the case of PSMA, it is expressed only within the cytoplasm of healthy prostate tissue and therefore remains unaffected by ADCs that target extracellular PSMA in prostate cancer cells [54]. On the other hand, ADCs targeting the HER2 receptor require the antigen to be highly overexpressed in breast cancer cells in comparison with healthy cells in order for the normal tissue to remain unaffected by the drug.

The level of antigen expression required for effective ADC activity varies depending on the antigen in question. As mentioned earlier, HER2 antigens on breast cancer cells are required to be highly overexpressed for effective targeting [55]. In contrast, CD19 antigens targeted in B-cell lymphoma may be limited to as few as 30000 per cell in order to elicit ADC activity [56]. There is, however, a minimum requirement of approximately 10000

antigens per cell, in order to ensure the delivery of lethal quantities of the cytotoxic drug [57]. The variability in the prerequisite number of targets emphasizes the need to use preclinical tumour models that are similar to human tumours with respect to antigen expression levels. An added complication arises from the fact that the initial estimate of antigen expression does not stay constant, but instead varies during the course of the treatment [58].

In addition to specific and sufficient expression, an optimal target antigen should also incite effective ADC internalization [51]. The rate and efficiency of internalization depends on the type of target and the choice of cytotoxin. Some targets internalize frequently regardless of ligand binding, whereas others reside permanently on the cell surface. Although it was initially thought that ADCs targeting non-internalizing antigens would have poor efficacy [59], a study using rituximab bound to doxorubicin/auristatin showed it underwent efficient internalization despite binding to the non-internalizing antigen CD20. This was however, not the case when the same mAb was conjugated to a different cytotoxic drug, calicheamicin [39]. The internalization efficiency of some antigens also depends on the specific epitope that binds to the mAb, as this leads to varying levels of antigen–antibody affinity [60,61].

Some ADCs are capable of eliciting antigen-mediated anti-tumour activity in addition to the cytotoxic activity arising from the conjugated drug. This is possible when the target antigen has a biological role in cancer pathways that is subsequently inhibited by the binding of an antibody. The most commonly cited example of an ADC with proven anti-tumour activity resulting from the target antigen is T-DM1 [62]. Trastuzumab is a humanized antibody that targets the transmembrane tyrosine kinase receptor, HER2 [a member of the EGFR (epidermal growth factor receptor) family] that is commonly overexpressed in a quarter of all breast cancers [63]. Unlike most other receptors, HER2 has no known endogenous ligand but is instead activated by forming homo or heterodimers with other members of the EGFR family [64]. Once HER2 is activated, downstream effector molecules initiate intracellular (tumour-inducing) signalling pathways such as the phosphoinositide 3 kinase (PI3K) pathway (which prevents cellular apoptosis) [65] and the mitogen-activated protein kinase (MAPK) pathway (which promotes cell proliferation) [66].

Types of Tumour-Associated Antigens

Although cell-surface proteins are the most commonly used targets for ADCs, there are various other categories of tumour-associated antigens including glycoproteins, proteins of the extracellular matrix and aberrant gangliosides on the tumour cell surface [67,68]. Apart from antigens found on tumour cells, there is a growing interest in targeting antigens present on tissues that support the growth and spread of tumour cells such as neovasculature or extracellular stromal tissue [69–73]. This is particularly attractive as these tissues have a stable genome unlike cancer cells and are therefore less likely to undergo somatic mutations, reducing the risk of mutation-mediated drug resistance. Instead, non-tumour tissue targets differentiate themselves from healthy tissue by being in an undeveloped state as



a result of their rapid formation and turnover rate [74]. ADCs that bind to neovasculature destroy the tumour blood supply and cause tumour cell death via nutrient deprivation and hypoxia [75,76]. Potential targets in tumour vasculature include vascular endothelial growth factor (VEGF) and its receptors integrin and endoglin. ADCs that target and destroy extracellular stromal tissue cause tumour cell death by reducing the concentration of growth factors produced by stroma. Similar to tumour vasculature, these growth factors are critical in promoting tumour cell survival [77]. Examples of anti-tumour stromal targets include fibroblast activation protein (FAP) and protein tyrosine kinase 7 (Ptk 7), a pseudokinase enzyme commonly found on many cancer and stromal cells [78]. Since all tumours depend on angiogenesis and stromal factors for their survival and growth, ADCs that target such tissues have a wider efficacy.

Choosing Monoclonal Antibodies

mAbs allow ADCs to have high target-specificity, target-affinity and prolonged drug exposure at the tumour site. Based on these features, antibody selection should ideally ensure minimal cross-reactivity with healthy tissues, sub-nanomolar affinity to the target antigen and a long pharmacokinetic half-life combined with minimal immunogenicity [79]. Over time, these features result in the accumulation of the ADC at the tumour site and allow it to have an increased therapeutic effect. In addition, it is beneficial for the mAb to possess intrinsic anti-tumour activity resulting from either direct modulation of the biological activity of the target antigen (e.g., anti-HER2 mAbs) [80] and/or via immune effector functions such as ADCC, CDC and CDCC [81]. When constructing the ideal ADC, it is important to maximally preserve the favourable properties of the mAb.

With regard to tumour specificity and target affinity, it is important to choose a mAb that does not lose these features through non-specific binding to normal cells. Apart from being toxic to healthy tissue, an antibody lacking tumour specificity may be eliminated from the circulation due to immunogenicity, leading to sub-optimal target exposure and decreased therapeutic effect [82]. The complementarity-determining regions of an antibody (i.e. the antigen-binding sites) should have extremely high (preferably sub-nanomolar) target affinity in order to guarantee efficient internalization [83].

The immunogenicity of an ADC is one of the major determinants of its circulatory half-life. Early ADCs made use of murine mAbs that evoked a strong, acute immune response in humans that resulted in the rapid formation of human anti-mouse antibodies within 2 weeks of a single dose [84]. Since then, murine mAbs have been replaced with chimeric IgG antibodies that have a human constant region and a murine variable region [85]. Another alternative is the use of humanized IgG antibodies that have a completely human variable sequence except for the portion responsible for antibody-antigen complementarity [86]. Most ADCs that are currently in use or in clinical development employ either humanized or fully human antibodies [19]. Brentuximab vedotin (an anti-CD30 ADC) [34,87] and BT062 (an anti-CD138 ADC) both incorporate chimeric mAbs [88].

A major benefit of using mAbs and mAb-based drugs, such as ADCs, is their favourable pharmacokinetics with respect to distribution, metabolism and elimination in comparison with small-molecule cancer therapies. Once mAbs are administered into the bloodstream, they can distribute into cancer tissue either via extravasation through pores in the endothelium or via pinocytosis through endothelial cells following a diffusion gradient [89]. The distribution of the ADC (and hence the cytotoxic drug) into tumour tissues is limited by the size of the antibody, which represents more than 90 % of the mass of an ADC [90]. However, unlike normal blood vessels that have a monolayer of endothelial cells forming tight junctions with one another, tumour endothelium is characterized by excessive branching and sprouting, resulting in a leaky monolayer [91]. The large size of ADCs therefore does not necessarily restrict their distribution into tumour tissue but minimizes their distribution into metabolizing and eliminating organs such as the liver, intestines, muscle and skin, thereby extending their half-life [92–94]. An additional mechanism by which ADC half-life is prolonged is through the binding of the Fc portion of humanized IgG antibodies to receptors within endosomal vesicles of endothelial cells known as human FcRns [95]. The resulting FcRn-ADC complex can either be transported back into systemic circulation or into the interstitial fluid surrounding tumour cells. FcRns therefore act as a useful reservoir for ADCs following the pinocytosis of the mAb into the endothelial cell. As the concentration of FcRns within endothelial cells is very high [96] and probably exceeds that of the internalized ADCs, their transport out of the cell is extremely efficient and prevents the ADC from causing unwanted endothelial cell death. Extensive research is being conducted to improve the binding of ADCs to endothelial FcRn receptors and thereby prolong their circulatory half-life [97].

The anti-tumour activity of mAbs may occur by both direct and indirect mechanisms [20]. Certain mAbs such as trastuzumab or rituximab exert direct cytotoxic effects by blocking the biological signalling activities of tumour antigens associated with cell-function and proliferation [98,99]. Other antibodies have indirect effects by promoting natural anti-tumour immune response mechanisms such as ADCC, CDC or CDCC (Figure 4). In ADCC, immune effector cells, such as macrophages and natural killer (NK) cells, bind to the CH3 region of IgG mAbs via their FcγRIIIa receptors [100]. Once bound, the immune cells become activated and mediate tumour cell killing via phagocytosis by macrophages or via the release of toxic granules from NK cells [101]. In CDC, the binding of a mAb to its target antigen on tumour cells triggers the classical complement pathway involving up to 30 circulating plasma proteins. The complement pathway begins with the binding of C1q to the second constant heavy chain (CH2) region of mAbs, resulting in the activation of a proteolytic cascade that terminates with the formation of a membrane attack complex. This complex results in tumour cell death via the action of pore-forming structures that release cellular contents [102]. In CDCC, another protein formed during the complement cascade, C3b, acts as an opsonin and interacts with C3b receptors present on NK cells and macrophages to facilitate tumour cell lysis. As all the effector functions of mAbs,

require the binding of the constant region to various receptors, they work best when the target antigen undergoes limited internalization.

The strength of the various immune effector functions varies depending on the specific isotype of monoclonal IgG antibodies used in the ADC. The effects of ADCC and CDC are much stronger in human IgG1 and IgG3 isotypes in comparison with IgG4 and IgG2 mAbs [104,105]. Although efficient at tumour cell lysis, the short circulation half-life of IgG3 antibodies compared with IgGs 1, 2 and 4 makes them a poor choice for use in ADCs [104,106]. IgG4 antibodies are also not preferred due to their tendency to exchange one-half of themselves with another IgG4 antibody with the possibility to form a new hybrid IgG4 mAb. In order to overcome this, the CH3 region of IgG4s, responsible for the formation of hybrid antibodies, can be replaced with the CH3 region of IgG1 mAbs [107]. IgG2 antibodies are favoured for therapeutic use due to their tendency to form covalent dimers that aid antibody-antibody associations. These associations have the potential to enhance the affinity and/or internalization of the mAb but may also cause the ADC to aggregate and become ineffective [108]. The difference in the extent of immune effector function is evident in previously approved ADCs that show substantial variation with regard to intrinsic anti-tumour activity. Gemtuzumab ozogamicin, for example, uses an IgG4 mAb that lacks any effector activity, [32] whereas brentuximab vedotin that is based on an IgG1 sub-type shows much better (although still modest) ADCC action [109].

Enhanced antibody functionality may be achieved by employing bispecific antibodies engineered to comprise two different antigen-binding sites capable of recognizing two different epitopes. These can be on the same antigen or on different antigens, usually on the surface of two different cells [110]. The first therapeutic bispecific antibody, catumaxomab, was approved in Europe in 2009 and targets EpCAM antigens (on tumour cells) and CD3 (on T-cells). In 2014, the US FDA gave accelerated approval for a bispecific antibody blinatumomab, designed based on bispecific T-cell engager (BiTE) immunotherapy. Blinatumomab binds CD19 on the surface of B-cell lymphoblasts and CD3 on the surface of T-cells, thus bringing them together and enabling T-cell mediated killing of the tumour cells. More bispecific antibodies are in clinical development for oncology, autoimmune and infectious-disease indications [111]. A variety of bispecific antibodies with tumour-specific antigens such as HER2/neu, CEA (carcinoembryonic antigen) and CD30 have all been clinically validated. The design of bispecific antibodies is however complicated by the need to engineer multiple antigen-binding domains into a single construct.

Although mAbs can now be engineered to have increased circulation half-lives and improved immune effector functions [112,113], there is limited clinical data regarding the use of such optimized antibodies for enhanced ADC function. The notion that ADCs with high target affinity possess high cytotoxic capacity is, for example, yet to be demonstrated in preclinical models [114] or in the clinic. Goldmacher and Kovtun [38] have hypothesized that such a correlation may in fact not exist, as ADCs with high target affinity may rapidly bind to perivascular regions of the

tumour rather than bind uniformly to all tumour cells [115,116]. Similarly, the assumption that ADCC and CDC contribute to the anti-tumour activity of ADCs [104,105] might prove to be incorrect as these effector functions are generally weak and compete with ADC internalization. In fact, IgG2 and IgG4 isotypes of antibodies that have poor immune effector function for naked mAbs were the preferred antibodies for use in certain ADCs [30,117,118]. The benefit of using fully human antibodies as opposed to chimeric and humanized versions has also not been demonstrated in clinical trials, as patients with advanced cancers lack the ability to produce antibodies against therapeutic mAbs [90]. These poorly established criteria for antibody selection and their impact on ADC activity make it difficult to design an optimized mAb.

Linkers

Linker chemistry is an important determinant of the safety, specificity, potency and activity of ADCs. Linkers are designed to be stable in the blood stream (to conform to the increased circulation time of mAbs) and labile at the cancer site to allow rapid release of the cytotoxic drug [119]. There are various parameters that are taken into consideration when designing the ideal linker. These include the cleavability of the linker and the position and mechanism of linkage (i.e. conjugation chemistry) [42]. Linkers are traditionally classified based on the first parameter, i.e. the mechanism by which they release the cytotoxin [51]. Existing linkers belong to either one of two broad linker designs: cleavable or non-cleavable linkers.

Cleavable linkers exploit the differences between conditions in the blood stream and the cytoplasmic conditions within cancer cells [120]. The change in environment once the ADC-antigen complex is internalized, triggers cleavage of the linker and release of the active drug, effectively targeting toxicity to cancer cells. There are three types of cleavable linkers: hydrazone, disulfide and peptide linkers, each of which responds to different cancer-specific intracellular conditions. Hydrazone linkers were among the first to be developed and depend on the low pH within lysosomes to undergo acid hydrolysis and release the cytotoxic drug [121]. Gemtuzumab ozogamicin is an example of an ADC that used acid-labile hydrazone linkers. Unfortunately, the stability of such linkers came under scrutiny [33] when results from a clinical trial showed that gemtuzumab ozogamicin was associated with significantly higher toxicity in comparison with standard therapy [31]. An alternative to hydrazone linkers is the use of disulfide linkers. These take advantage of elevated concentrations of thiol molecules (e.g., glutathione) within cancer cells. Thiol molecules are especially high in tumours, as they are involved in promoting cell-survival and tumour growth and are produced during cell-stress conditions such as hypoxia [122]. The third type of cleavable linker is the enzyme labile or peptide linker. In comparison with hydrazone and disulfide linkers, peptide linkers offer improved control of drug release by attaching the cytotoxic drug to the mAb via a dipeptide linkage [123]. The proteases required to break the peptide bond are only active in low pH environments, making it highly unlikely that the cytotoxic drug

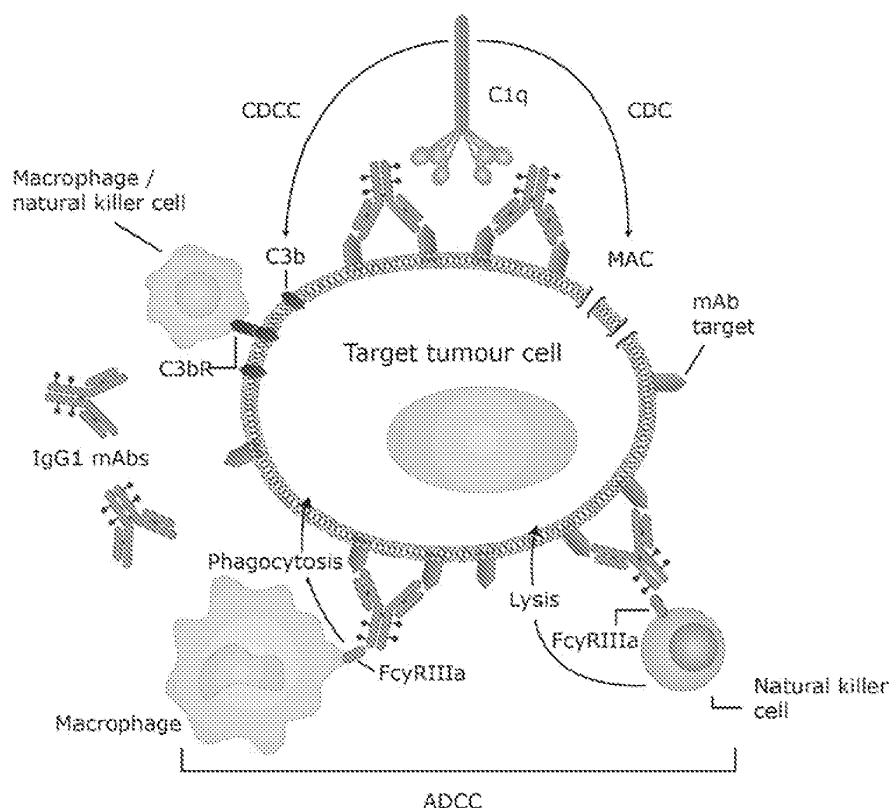


Figure 4 Mechanism of ADCC, CDCC and CDC [103]

is released in the pH-neutral environment of the blood. Instead, the dipeptide linkage is cleaved in the acidic environment within lysosomes by lysosomal proteases, such as cathepsin-B and plasmin [124]. Brentuximab vedotin is an example of an ADC that employs a dipeptide linkage consisting of valine and citrulline along with a para-aminobenzylcarbamate spacer molecule that separates the large cytotoxic drug from the mAb [125]. A clinical trial comparing the three cleavable linkers showed that enzyme-labile linkers had lower *in vivo* toxicity as a result of greater specificity, increased stability and a longer half-life compared to hydrazone linkers [126].

In contrast with cleavable linkers that are reliant on distinctive intracellular conditions to release the cytotoxin, non-cleavable linkers depend solely on the process of lysosomal degradation following ADC-antigen internalization. Protease enzymes within the lysosome breakdown the protein structure of the mAb, leaving behind a single amino acid (usually a lysine or a cysteine) still attached to the linker and cytotoxin. The resulting amino acid-linker-cytotoxin complex is released into the cytoplasm and subsequently becomes the active drug. In comparison with cleavable linkers, non-cleavable linkers were found to have improved stability in the bloodstream allowing ADCs with such linkers to

have longer half-lives and pose a reduced risk from side effects while retaining the activity of the cytotoxic drug [127]. This knowledge was reflected in the clinical development of T-DM1, originally designed to have a valine-citrulline dipeptide linker but was instead produced with a non-cleavable thioether linker [128]. Despite the successful use of non-cleavable linkers, it is important to note that their dependence on lysosomal degradation means they can only be used in ADCs targeting antigens that undergo efficient intracellular internalization.

Once an optimal linker for a particular ADC is chosen, it is important to determine the ideal number of drugs to be conjugated to a mAb [i.e., the drug-antibody ratio (DAR)]. Because of potential linker instability and poor ADC internalization, several drugs are required to be linked to each mAb to achieve adequate cytotoxicity. On the other hand, excessive drug conjugation might result in increased clearance and/or immunogenicity as the ADC is more likely to aggregate and be recognized as a damaged, malformed or foreign protein [129]. In a study conducted by Hamblett et al. [130], the *in vivo* efficacy of ADCs containing four drugs per antibody was found to be equivalent to that of ADCs with eight drugs per mAb. Heavily modified ADCs (i.e., those with eight conjugated drugs) were associated with increased

toxicity and underwent faster clearance from the circulation. Additionally, it was found that ADCs containing only two drugs per mAb had a much wider therapeutic window and therefore a higher maximum tolerated dose. The study consequently concluded that ADCs loaded with 2–4 drugs per mAb achieved the best balance between slow clearance and maximal potency [130].

A critical aspect of linker function is the presence or absence of a phenomenon known as the bystander effect. This effect refers to target-cell mediated killing of healthy cells neighbouring the tumour cell. The bystander effect is generally caused by cellular efflux of hydrophobic cytotoxic drugs, capable of diffusing out of an antigen-positive target cell and into adjacent antigen-negative healthy cells. Although the bystander effect undermines the target specificity of ADCs, it can be advantageous when tackling solid tumours that lack homogenous expression of the target antigen [131]. However, the bystander effect is only observed with the use of cleavable linkers, as the amino acid-linker-cytotoxin complex formed from the breakdown of non-cleavable linkers is charged and therefore not capable of crossing the hydrophobic lipid bilayer of the target cell [132]. Cleavable linkers are therefore associated with wider efficacy as they can take effect independent of internalization. However, the extent to which they can cause bystander cell death depends largely on conjugation chemistry [133]. Regardless of the extent of cytotoxicity, non-selective ADCs with cleavable linkers are only viable if the bystander effect is restricted to a small number of non-target cells.

Conjugation Chemistry

In recent years, a great deal of research has been conducted in order to develop novel conjugation techniques for use in future ADCs. Traditionally, cytotoxic drugs have undergone chemical conjugation to mAbs, whereby reactive portions of native amino acids are made to interact and bind a specific part of the linker molecule [134]. Examples of reactive groups include the epsilon-amino end of lysine residues (used in the conjugation of T-DM1) [135] and the thiol side chains present in the partially reduced form of cysteine residues (used in brentuximab vedotin) [136]. As this technique relies on native amino acids, conjugation of the drug is limited by the peptide sequence of the antibody, which therefore restricts control over the number and position of attached cytotoxic drugs. The resulting heterogeneous ADC mixture is a major drawback to the chemical conjugation technique as it impacts the toxicity, stability and potency of the ADC.

Heterogeneity, with respect to the number of cytotoxin molecules attached per antibody (Figure 5), results in only a small portion of the prepared ADC solution being therapeutically active. This is because a subset of ADCs will contain too few cytotoxin molecules to retain their cell-killing capacity whereas others will have too many to maintain their stability in the bloodstream [137]. Furthermore, inactive ADCs directly decrease ADC potency by binding the limited number of target antigens available on tumour cells and blocking the binding of therapeutically-active ADCs.

Heterogeneity, with respect to the position of the cytotoxin on the mAb, plays an important role in the therapeutic effect of

ADCs. In an experiment conducted by Shen et al. [139], ADCs with a cytotoxin conjugated at the light chain of the mAb were shown to have significantly higher *in vivo* efficacy compared with heavy chain conjugates.

Currently researched methods for the development of site-specific drug conjugation are based on introducing selectively reactive molecules at specific locations along the mAb. This allows more control over the site and number of drug attachments and can potentially increase the therapeutic window of ADCs, while reducing toxicity and improving pharmacokinetics [140]. The three alternatives to conventional conjugation techniques include conjugation via novel unpaired cysteine residues conjugation via transglutaminases and, finally, conjugation via unnatural amino acids.

Conjugation of novel unpaired cysteine residues to a small portion of the mAb relies on site-directed mutagenesis to introduce a fixed number of engineered cysteines at specific, controlled sites along the mAb [141,142]. This process maintains existing disulfide bonds between native cysteine residues while avoiding the problem of ADC heterogeneity. A study conducted by Junutula et al. [143] showed that T-DM1, when conjugated with unpaired cysteine residues, was as efficacious as traditional T-DM1, albeit with fewer side effects and therefore a wider therapeutic window. This technique is however associated with several drawbacks as the newly introduced, unpaired cysteine residues are at risk of reacting with native cysteine amino acids and creating malformed disulfide bridges that could potentially disrupt the binding capacity of the mAb [144]. There is also the possibility of the unpaired cysteine residues reacting with nearby cysteines on neighbouring mAbs and producing an antibody dimer that could again destabilize the mAb and undermine its functionality [145]. Given these potential problems, this technique is best used when specific sites are identified on the mAb where the new cysteine residues are unlikely to react with other amino acids.

A second method of site-specific drug conjugation uses a microbial transglutaminase enzyme in order to conjugate an amine-containing cytotoxic drug to a mAb modified to have a specific number of glutamine side chains [146,147]. A major advantage of the microbial transglutaminase technique is that it does not recognize pre-existing, native amino acids, [148] but instead interacts with glutamine 'tag' sequences that can be incorporated into the mAb via plasmids. This technique allows better control over drug conjugation due to an increased number of possible conjugation sites on the mAb. A study conducted by Strop et al. [149] used microbial transglutaminase to conjugate an EGFR targeting mAb and an anti-MSI1 (chromosome 1, surface marker 1) antibody to the cytotoxic drug monomethyl dolastatin 10. There were several sites of successful conjugation in both conjugates with both of them displaying strong *in vivo* (and *in vitro*) activity. Apart from transglutaminases, other enzymes such as glycotransferases- and formylglycine-generating enzymes have also been investigated for use in a similar manner [150,150].

The final method of drug conjugation is based on the use of non-natural amino acids, such as selenocysteine or acetylphenylalanine [151,152]. These are structurally very similar to their natural amino acid counterparts with only

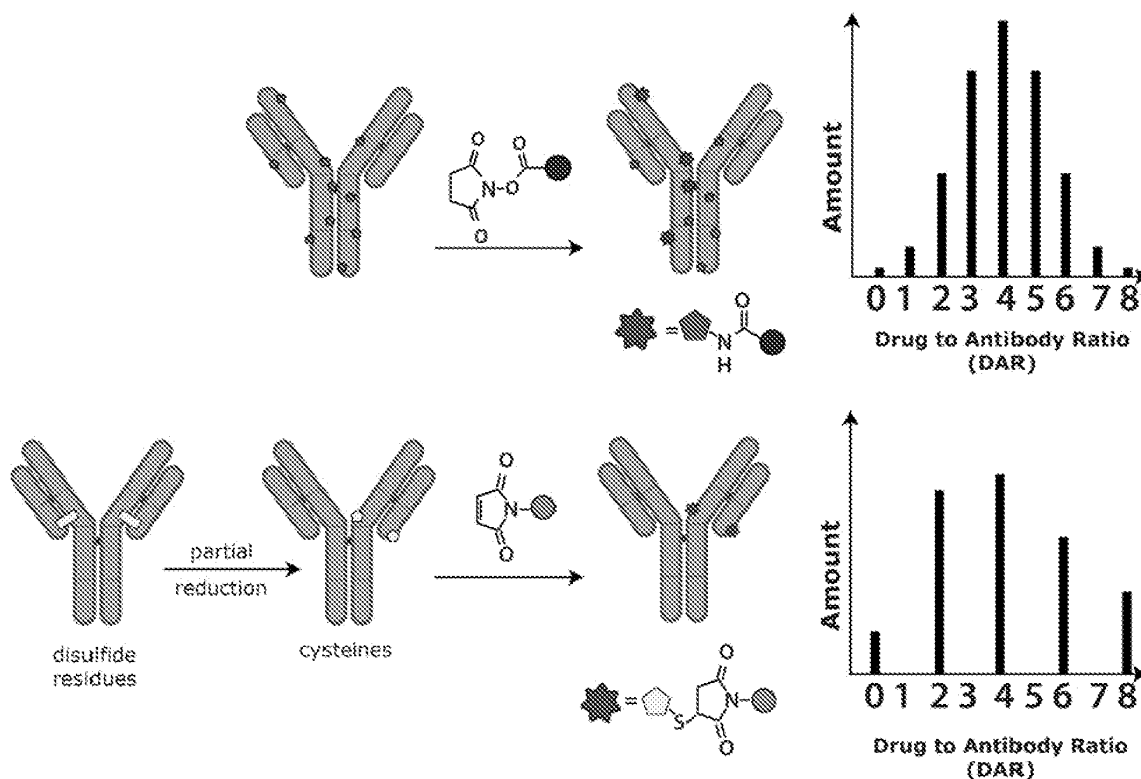


Figure 5 Mechanisms of traditional conjugation with DARs [158]

minor differences in functional groups. Selenocysteine, for example, differs from the traditional cysteine residue by substituting a sulfur atom with a selenium atom. Similarly, acetylphenylalanine contains a ketone group that is not present on any of the 20 naturally occurring amino acids [153]. These small differences allow site-specific modification while sparing the pre-existing residues of the mAb. Unnatural amino acids are introduced into the mAb during transcription with the use of stop codons paired to the insertion sequences of selenocysteine or acetylphenylalanine [154]. Once the unnatural amino acid is introduced into the mAb, it is available for conjugation with a suitable cytotoxin, which, in the case of nucleophilic selenocysteine [155] has to be a positively charged molecule. A study that examined the binding of a mutant anti-HER2 mAb containing acetylphenylalanine found that its affinity for its ligand, the HER2 receptor, was comparable to T-DM1 [156]. In addition to the two unnatural amino acids mentioned, there are several others that are currently being developed that may be introduced to the mAb during *in vitro* transcription and translation [157].

Cytotoxins

As ADCs are most often prepared in an aqueous solution and administered intravenously, it is important that the cytotoxic drug has prolonged stability in such environments to prevent damage

to healthy cells and increase the availability of the drug at the tumour site. Similarly, it is important that the molecular structure of the cytotoxin allows for its conjugation to the linker while avoiding immunogenicity, maintaining the internalization rate of the mAb and promoting its anti-tumour effects (i.e., ADCC, CDCC and CDC) [46]. Regardless of the stability of the cytotoxin, only a small portion of the administered ADC reaches the tumour cells. This makes it imperative that the conjugated cytotoxic drug is potent at low concentrations. Typically, the cytotoxins used in ADCs are a 100–1000 times more potent than regular chemotherapeutics and preferably have sub-nanomolar potency [158]. Most classes of cytotoxins act to inhibit cell division and are classified based on their mechanism of action. Since many ADCs utilize cytotoxins that target rapidly dividing cells, there is a decreased risk of unwanted toxicity if the ADC mistakenly delivers the drug to a non-replicating cell. As the cytotoxin is most commonly released in the lysosome following cleavage of the linker molecule, it is important to ensure that the cytotoxin remains stable in low pH environments and has the capacity to move into the cytosolic or nuclear compartments of the cell within which it takes effect. The choice of the specific cytotoxin to be used in an ADC depends on its mechanism of action and the type of cancer.

First generation ADCs made use of early cytotoxins such as the anthracycline, doxorubicin or the anti-metabolite/antifolate agent, methotrexate [159,160]. Although these cytotoxins worked

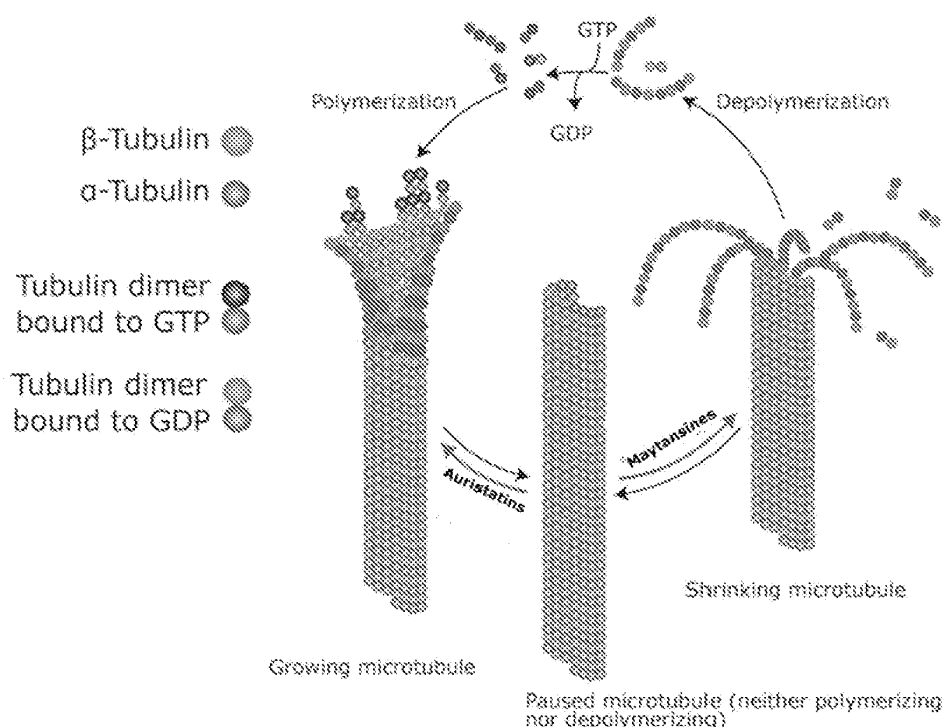


Figure 8 Effect of auristatins and maytansines on microtubule formation [138, 173]

well when administered as standard chemotherapy, they proved to have insufficient potency at low concentrations when conjugated to a mAb [161]. Current cytotoxins have far greater potency and can be divided into three main groups: auristatins, maytansines and calicheamicins. The former two both target rapidly dividing cells by interfering with different parts of the cell cycle [162,163] whereas calicheamicins, along with the less commonly used groups of cytotoxins: duocarmycins and pyrrolbenzodiazepine (PBD) dimers, all induce DNA damage [164]. Regardless of the cell-killing mechanism, all five categories of cytotoxins result in cancer cell death by induction of apoptosis [165].

The auristatins, monomethyl auristatin E (MMAE) and monomethyl auristatin F (MMAF) are specific types of mitotic inhibitors that share their mechanism of action with the traditionally used taxane chemotherapeutics. Auristatins interfere with the formation of microtubules by binding to the β -subunit of α - β tubulin dimers in the cytoplasm. The drug subsequently takes effect by preventing the hydrolysis of GTP molecules on the β -subunit, causing continuous and excessive growth of microtubules (Figure 6). As the microtubules lose their capacity to shorten and separate sister chromatids during anaphase, the cell becomes frozen in the metaphase portion of mitosis [166]. MMAE is used in brentuximab vedotin as well as several other ADCs currently under clinical development [167,168]. Since MMAE is hydro-

phobic, it can easily diffuse out of the target cell and mediate the killing of nearby bystander cells [169]. This might be a potential drawback for the use of MMAE in ADCs targeting non-solid haematological cancers with homogenous antigen expression.

Similar to auristatins, maytansines, the derivatives of which are known as maytansinoids (DMs), also interfere with microtubule assembly but are mechanistically similar to vinca alkaloids [170]. Maytansines take effect by binding and capping the 'plus' end of the growing microtubule and block the polymerization of tubulin dimers preventing the formation of mature microtubules. Existing microtubules further disassemble once the GTP molecule on the β -subunit (of the α - β tubulin dimer) is hydrolysed, which again freezes the cell in metaphase preventing cell division (Figure 6) [171]. Of the two DMs, DM1 and DM4, the former is used as the active drug in T-DM1 [172].

Calicheamicins, duocarmycins and PBD dimers are all different types of DNA-damaging agents that are functionally similar to anthracyclines in that they all target the minor groove of DNA [174–176]. The DNA double helix forms two grooves which are present as a result of the geometric conformation of the two anti-parallel strands [177]. The minor groove is narrower and consists of fewer exposed base pairs in comparison with the major groove. Calicheamicin is the extremely potent anti-tumour antibiotic used in gemtuzumab ozagamicin [178]. It binds the minor grooves of tumour cell DNA where it forms reactive diradical species that



ultimately cause cleavage of the DNA strands at various locations [179]. Duocarmycins and PBD dimers take effect in similar ways with the former acting as a DNA minor groove alkylating agent and the latter as a minor groove cross-linker [180,181]. As with any normal tissue, damage to the DNA of a cancer cell induces cell death via apoptosis.

CLINICAL DEVELOPMENT OF ADCs

Many lessons have been learnt since the FDA withdrawal of gemtuzumab ozogamicin regarding the design and development of ADC components. Pharmaceutical companies investing in ADC research have made significant advances in linker technology, conjugation chemistry, antibody engineering and the identification of potent cytotoxins, resulting in rapid evolution of the field. This has not only led to the recent approvals of brentuximab vedotin and trastuzumab emtansine but has also driven the clinical development of ADCs. Currently approved ADCs and those in advanced clinical development are listed in Table 1.

One particularly promising ADC, inotuzumab ozogamicin (Pfizer), has recently been discontinued for the treatment of aggressive non-Hodgkin's lymphoma following the failure of a once-a-month dose of the drug with rituximab to meet the primary objective of improving overall survival. This was in comparison with a combination of bendamustine/gemcitabine with rituximab. However, it continues to be investigated in phase 3 for acute lymphoblastic leukaemia, recognized in the U.S. and the E.U. as an orphan indication with few other treatment options [182].

Despite the strong ADC pipeline, the cost of ADC development remains a major drawback in terms of their widespread use in the clinic. The current cost of treatment with the mAb trastuzumab, is approximately \$767 per dose [183], with the price almost doubling following conjugation to a cytotoxin. The production of ADCs, in particular, is especially expensive due to the incorporation of conjugation technology that complicates their manufacturing process. Research is currently directed at decreasing the costs of ADC production with the development of cheaper techniques to produce mAbs such as the use of algae as a vector for protein expression [184].

DISCUSSION: CHALLENGES AND FUTURE OF ADCs

The major challenges associated with the development of ADCs arise from factors that interfere with ADC efficacy and/or those that result in ADC-mediated non-target cell toxicity. All three components of an ADC contribute to these challenges and need to be optimized to create a successful conjugate. Once the therapeutic effect of an ADC has been maximized, it is also desirable

to prolong these effects by avoiding resistance mechanisms that decrease the duration of ADC efficacy. All targeted cancer therapies (including ADCs) are prone to resistance mechanisms that alter the function of the target antigen and render the treatment redundant [186]. The future of ADCs will mainly depend on our ability to tackle these challenges.

With more research being conducted on the use of various targeted therapies, there has been a considerable increase in the number of target antigens viable for antagonism by ADCs [187]. A selective process may therefore be employed when choosing a target that is not only widely expressed and tumour-specific but also displays minimal susceptibility to mutations and therefore to resistance. To prevent resistance and ensure the long-term use of any targeted therapy, the target antigen and the signalling pathway it triggers have to remain stable during the treatment period. Unlike traditional chemotherapeutic agents that are intrinsically non-specific and have poorly understood mechanisms of action, the targeted nature of ADCs allows better understanding of resistance mechanisms [188]. The specific type of resistance varies depending on the type of tumour antigen (EGFR, HER2) and the tumour pathway (RAS [rat sarcoma]-RAF [rapidly accelerated fibrosarcoma]-MAPK, PI3K-AKT) and can be either genetic (e.g., mutations in target antigen/downstream proteins) [189] or non-genetic (e.g., activation of compensatory signalling) [190]. Further investigation into other routes of resistance may allow the development of new drugs capable of overcoming resistance.

With regard to mAbs, lack of specificity to the target antigen might result in cytotoxic effects on healthy tissue, whereas poor internalization or inadequate antigen-binding affects the therapeutic effect of the ADC. In addition to these challenges, the large size of mAbs limits their capacity to penetrate tumour tissues, which therefore complicates the use of ADCs against solid tumours. In order to combat this, current research has explored the use of antibody fragments such as diabodies and minibodies (Figure 7) which, by virtue of their size, have better tumour penetration but shorter half-lives in comparison with standard mAbs [191,192]. The size of diabodies also allows better tumour binding as the number of antigen-binding sites per unit area is increased. Although the clinical efficacy of diabodies is yet to be demonstrated, their potential application in cancer treatment has been proven in several preclinical models [193–195]. However, as previously outlined, the current optimization criteria for ideal mAbs need not necessarily translate into improved ADCs. As more clinical data emerges, it might be possible to identify clearer principles for developing mAbs in the context of ADCs.

The future of linker chemistry is moving strongly toward the identification of new conjugation techniques that yield homogenous ADC mixtures. Research within this area has greatly expanded in the past few years with the identification of new techniques to attach cytotoxic drugs to antibodies.

Despite advances in many aspects of ADC development, the list of amenable cytotoxins for use in ADCs has remained relatively short [197]. This is an indication of the challenges involved in finding soluble, linkable drugs that are potent enough to allow for the optimal DAR of 2–4 drugs per antibody [130]. Cytotoxins such as

Table 1. *ADCs in the market and in late clinical development [183]*

ADC	Sponsor	Indications	Target antigen	Antibody type	Linker	Cytotoxin	Status/Phase
Genituzumab ozogamicin	Pfizer	AML	CD33	Humanized IgG4	Acid-labile hydrazone 4-(4-acetylphenoxy) butanoic acid	Calicheamicin	FDA approved in 2000. Withdrawn in 2010
Brentuximab vedotin	Seattle Genetics	Relapsed Hodgkin lymphoma and systemic anaplastic large cell lymphoma	CD30	Chimeric IgG1	Cathepsin cleavable valine-citrulline	MMAE	Accelerated approval by the FDA in 2011
T-DM1	Genentech	Relapsed or chemotherapy refractory HER2-positive breast cancer	HER2	Humanized IgG1	N-succinimidyl 4-(N-maleimidomethyl) cyclohexane-1-carboxylate (SMCC)	DM1	FDA approved in 2013
Inotuzumab ozogamicin	Pfizer	Aggressive non-Hodgkin's lymphoma (stopped) Acute lymphoblastic leukaemia	CD22	Humanized IgG4	Acid-labile hydrazone (4-(4-acetylphenoxy) butanoic acid)	Calicheamicin	III
Pinatuzumab vedotin (RG-7593)	Genentech	DLBCL and follicular non-Hodgkin's lymphoma	CD22	Humanized IgG1	Cathepsin cleavable valine-citrulline	MMAE	II
RG-7596	Genentech	DLBCL and follicular non-Hodgkin's lymphoma	CD79b	Humanized IgG1	Cathepsin cleavable valine-citrulline	MMAE	II
Lifastuzumab vedotin (RG-7593)	Genentech	Non-small-cell lung cancer; ovarian tumour	NaP2b	Humanized IgG1		MMAE	II
Gemtuzumab vedotin	Cellnex therapeutics	Breast cancer; melanoma	Glycoprotein NMB	Human IgG2	Cathepsin cleavable valine-citrulline	MMAE	II
Cofiximab Ravtansine (SAR-3419)	Sanofi	DLBCL; acute lymphoblastic leukaemia	CD19	Chimeric IgG1	Disulfide SPDB	DM4	II
Lenvotuzumab mertansine (IMGN-901)	Immunogen	Small-cell lung cancer	CD56	Humanized IgG1	Disulfide SPP	DM1	II
Indatuximab Ravtansine (BT-062)	BioTest	Multiple myeloma	CD138	Chimeric IgG	Disulfide SPDB	DM4	II
Anti-PSMA ADC	Progenics	Prostate cancer	PSMA	Human IgG1	Cathepsin cleavable valine-citrulline	MMAE	II
Labetuzumab-SN-38	Immunomedics	Colorectal cancer	CEA (also known as CD66e)	Humanized IgG1	Lysine	Irinotecan metabolite (SN-38)	II
MLN-0264	Takeda-Millennium	Gastrointestinal tumour; solid tumours	Quaril cyclase C	Human IgG	Protease cleavable	MMAE	II
ABT-414	AbbVie	Glioblastoma; non-small-cell lung cancer; squamous cell tumours	EGFR			MMAF	I/II
Milatuzumab doxorubicin	Immunomedics	Chronic lymphocytic leukaemia; multiple myeloma; non-Hodgkin's lymphoma	CD74	Humanized IgG1	Hydrazone	Doxorubicin	I/II

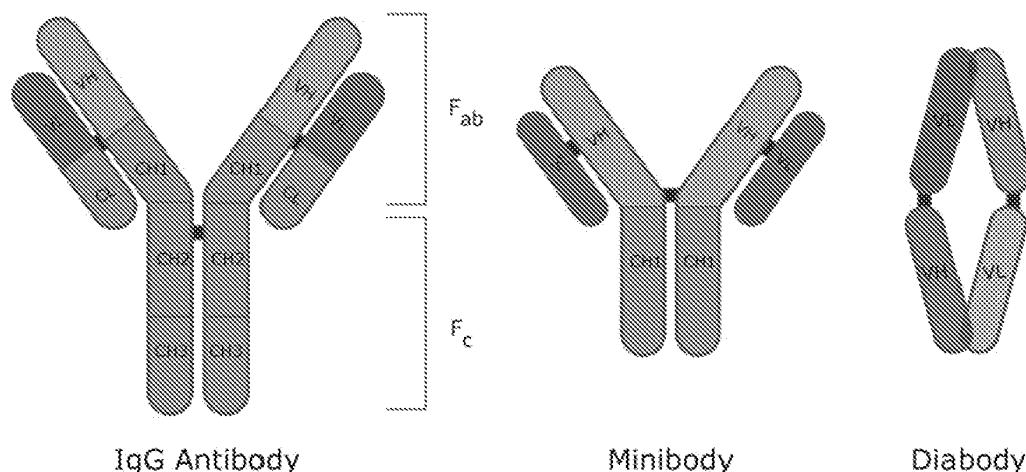


Figure 7 Structures of IgG antibody, minibody and diabody [198]

auristatins or maytansines that selectively target rapidly replicating cells are less susceptible to non-target toxicity in case the ADC mis-delivers the drug to non-dividing cells. On the other hand, DNA-damaging agents capable of causing apoptosis in all cells are more likely to be poorly tolerated and have far more side effects [47]. Clinical trials of ADCs that utilize both types of drugs have however shown that they are commonly associated with neutropenia or thrombocytopenia [198,199] which may in some cases limit their maximum dose [200]. As more cytotoxins are being identified, it is likely that the future of ADCs will produce a diverse range of drugs with different mechanisms of action and fewer side effects [201].

Most cytotoxins are susceptible to resistance via an array of mechanisms [202]. Resistance usually occurs through the overexpression of non-specific, active transporters, such as multi-drug resistance protein (MRP) or P-gp on cancer cells [203]. These transporters sense cell-damaging agents within tumour cells and expel them. As the active sites for both P-gp and MRP are found within the cell membrane of cancer cells [204,205], cytotoxins given in the form of ADCs are less affected by cellular efflux as they are internalized once bound to their antigen. A major priority in the development of ADCs has been to further reduce the effects of active transporters and thereby increase intracellular concentrations of the cytotoxic drug. The two ways by which this can be achieved are through the administration of adjuvant drugs that block P-gp and MRP or through the conjugation of a cytotoxin that is a poor substrate for these transporters [206,207]. One research group has developed a novel hydrophilic linker, that when conjugated to maytansine, was processed by the tumour cell to form an active cytotoxic drug that was a poor substrate to P-gp [208]. Other mechanisms of resistance include decreased activation of drug, enhanced expression of drug-metabolising enzymes, increased DNA repair and failure to apoptose following drug action [209].

Despite challenges in their design, ADCs have created a new paradigm for novel cancer chemotherapy. With the specificity of mAbs and the cytotoxic capacity of small molecule drugs, ADCs promise to be a large part of the future of precision medicine as well as combination treatment. As more clinical trials are conducted on existing ADCs, it should be possible to fine-tune the components of forthcoming conjugates and improve their therapeutic risk-benefit ratio. Finding new target antigens for solid tumours, improving the understanding of mAb activity and developing novel cytotoxin-linker pairs would all pave the way for a new generation of ADCs.

AUTHOR CONTRIBUTION

Christina Peters was responsible for the conception and initial drafting of the whole manuscript. Stuart Brown was responsible for initial critical review and both authors were responsible for the revised manuscript.

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