

Plakophilin-2: a cell-cell adhesion plaque molecule of selective and fundamental importance in cardiac functions and tumor cell growth

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Abstract Within the characteristic ensemble of desmosomal plaque proteins, the *armadillo* protein plakophilin-2 (Pkp2) is known as a particularly important regulatory component in the cytoplasmic plaques of various other cell–cell junctions, such as the composite junctions (*areae compositae*) of the myocardial intercalated disks and in the variously-sized and -shaped complex junctions of permanent cell culture lines derived therefrom. In addition, Pkp2 has been detected in certain protein complexes in the nucleoplasm of diverse kinds of cells. Using a novel set of highly sensitive and specific antibodies, both kinds of Pkp2, the junctional plaque-bound and the nuclear ones, can also be localized to the cytoplasmic plaques of diverse non-desmosomal cell–cell junction structures. These are not only the *puncta adhaerentia* and the *fasciae adhaerentes* connecting various types of highly proliferative non-epithelial cells growing in culture but also some very proliferative states of cardiac interstitial cells and cardiac myxomata, including tumors growing in situ as well as fetal stages of heart development and cultures of valvular interstitial cells. Possible functions and assembly mechanisms of such Pkp2-positive cell–cell junctions as well as medical consequences are discussed.

Keywords Adherens junctions · Myxomata · Cardiac tumors · Nuclear plakophilins · Plakophilin-2

List of abbreviations

Ab, mAb	Antibody, monoclonal antibody
AJ	Adherens junction
ARVC/D	Arrhythmogenic ventricular cardiomyopathy/dysplasia
As	Antiserum
CJs	Composite junctions
gp	Guinea pig
IF	Intermediate-sized filament
m	Mouse
PBS	Phosphate-buffered saline
Pkp	Plakophilin
RT	Room temperature

Introduction

Of the obligatory molecular ensemble components of desmosomes, the group of the plaque-bound plakophilins (Pkps), has been the last discovered. In vertebrate cells, this subfamily of the closely related members of the larger *armadillo* plaque protein family is represented by at least one of the three isoform proteins (Pkp1, Pkp2, Pkp3) in all desmosomes but desmosomes with two Pkps in near-equimolar amounts have also been described (for reviews, see, e.g., Bass-Zubek et al. 2009; Delva et al. 2010; Franke 2010; Godsel et al. 2004; Green et al. 2010; Hatzfeld 2007; Neuber et al. 2010; Schmidt and Jaeger 2005; Schmidt and Koch 2008; Schmidt et al. 1994). Pkp2 shows the most widespread occurrence in the desmosomes of all proliferative epithelial cells of normal tissues as well as of the tumors and of cell cultures derived therefrom, in meningiothelia and meningiomas (Akat et al. 2003, 2008) and in the composite junctions connecting cardiomyocytes (Borrmann et al. 2000,

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2006; Franke et al. 2006; Mertens et al. 1996, 1999). Pkp3 coexists in similar amounts in the desmosomes of many of these cell types, with the exception of, e.g., hepatocytes and cardiomyocytes (Bonné et al. 1999, 2003; Borrmann et al. 2000, 2006; Rickelt et al. 2009, 2010; Schmidt et al. 1999; for tumors, see, e.g., Furukawa et al. 2005; Kundu et al. 2008; for special negative effects on Pkp3, see Aigner et al. 2007).

By contrast, Pkp1 has been found in suprabasal, highly differentiated cell layers of stratified epithelia (Hatzfeld et al. 1994; Moll et al. 1997; Schaefer et al. 1993; Schmidt et al. 1994; reviews: Bass-Zubek et al. 2009; Neuber et al. 2010; Schmidt and Koch 2008) and has also been noted in certain types of cells of stratified squamous carcinomas (for references, see, e.g., Franke 2010; Mertens et al. 1999; Neuber et al. 2010; Papagerakis et al. 2003; Schwarz et al. 2006; Sobolik-Delmaire et al. 2007; South et al. 2003). For two of the Pkps, two prominent splice variants of the gene products have been determined (Hatzfeld et al. 1994; Heid et al. 1994; Mertens et al. 1996; Schmidt et al. 1994; see also Gandjbakhch et al. 2011). Finally and rather surprisingly, diffusible nuclear and cytoplasmic forms, including some rather stable functional complexes, have also been described for all three Pkps (e.g., Bass-Zubek et al. 2008; Bonné et al. 1999; Hofmann et al. 2006; Mertens et al. 1996, 2001; Mueller et al. 2003; Schmidt et al. 1997). Table 1 summarizes the molecular data of the presently known human Pkp splice variant forms and the chromosomal position of the three genes.

Antibodies (Abs) specific for cytoskeletal molecules and thus characteristic of certain cell types have become important in the development and refinement of cell type diagnoses of tumors, in particular metastases (for references, see, e.g., Folpe and Gown 2001; Franke et al. 1983; Garrod et al. 1996; Gown and Vogel 1984; Mertens et al. 1999; Miettinen 2003; Moll 1993; Moll et al. 1986; Parrish et al. 1986). However, in the field of molecular diagnoses of most non-epithelial tumors, including those located in or at the heart as well as those derived from heart tissues, the progress made so far is still rather modest. Thus, it is hoped that

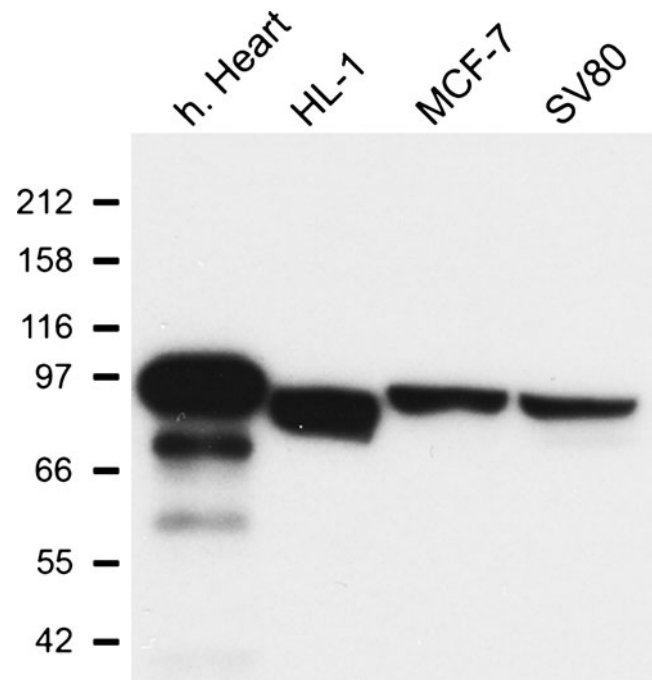


Fig. 1 Immunoblot specificity of the plakophilin-2 (Pkp2) antibodies used on polypeptides of total protein lysates of various mammalian cell cultures and human heart tissue. Immunoblot reaction of monoclonal murine antibodies (mAbs) specific for Pkp2 on SDS-PAGE-separated polypeptides present in lysates of cryostat sections of human heart tissue (*h. Heart*) (*lane 1*) or in lysates of permanently growing adult mouse cardiomyocyte-derived cells of line *HL-1* (*lane 2*), cells of the human breast adenocarcinoma-derived line *MCF-7* (*lane 3*) or SV40-virus transformed human fibroblasts of line *SV80* (*lane 4*). Note that the single polypeptide band of ca. 97 kDa appears not only in lysates of myocardial tissue and cardiomyocyte-derived cells but also of the epithelium-derived cells and in the transformed SV80 fibroblasts. Positions of polypeptide molecular weights are indicated on the left and correspond to 212, 158, 116, 97, 66, 55 and 42 kDa (from top to bottom)

new analytical insights into the cell type-specific components of the various cardiac cells will also contribute to the advancement of molecular diagnostics of cardiac tumors (for references, see McAllister and Fenoglio 1978; Miettinen 2003; Rickelt et al. 2010; Sheppard 2011).

Table 1 Molecular and biochemical characteristics of the presently characterized five prominent human plakophilin splice variant forms and the chromosomal position of the three genes

	Splice Variant	Accession number	Chromosome localization	Size of mRNA (bp)	No. of amino acids	Mol. wt (calc.)	Isoelectric point (calc.)	No. of arm-repeat units
Plakophilin-1	Pkp1a	NM_000299.3	1q32	5,384	726	80,496	9.18	9
	Pkp1b	NM_001005337.2		5,447	747	82,860	9.29	9
Plakophilin-2	Pkp2a	NM_001005242	12p11 ^a	4,307	837	92,756	9.35	9
	Pkp2b	NM_004572		4,439	881	97,415	9.39	9
Plakophilin-3	Pkp3	NM_007183	11p15	2,845	797	87,082	9.39	9

^a For Pkp2, a pseudogen at chromosome 12p13 has also been characterized (Bonné et al. 2000).

Materials and methods

Tissues and cell cultures

Samples from human heart tissue were obtained from the Department of Cardiac Surgery of the University Hospital Heidelberg, Germany (for details, see also Barth et al. 2009). Bovine as well as rat and mouse tissue samples, including fetal tissues, were freshly obtained from the regional slaughterhouse (Mannheim) or the laboratory animal facilities of the German Cancer Research Center (Heidelberg) as described (e.g., Franke et al. 2006). In addition, skin and heart tissue specimens from fetal German landrace pigs were provided from the Institute of Farm Animal Genetics (Friedrich-Loeffler-Institute, Mariensee, Germany). In general, the samples were fixed either with 4% formaldehyde in phosphate-buffered saline (PBS) and embedded in paraffin or snap-frozen in isopentane that had been precooled in liquid nitrogen and then stored at -80°C until use.

For the present study, cell cultures of the adult mouse cardiomyocyte-derived line HL-1 as well as various human

cell lines, including breast adenocarcinoma-derived line MCF-7, HaCaT keratinocytes, diploid lung fibroblasts of line WI38, SV40-virus transformed WI38 fibroblasts (line WI38VA13, subline 2RA) and SV40-virus transformed fibroblasts of line “SV80” were kept and used as described (e.g., Barth 2011; Barth et al. 2009; Claycomb et al. 1998; Pieperhoff et al. 2011; Rickelt et al. 2009). For comparison, bovine dermal fibroblasts of line B1, mouse 3T3 embryonic mesenchymal cells and the “junction-lacking” murine fibroblasts of strain L929 were examined in parallel.

Antibodies

The murine monoclonal antibodies (mAbs) and polyclonal guinea pig Abs specific for plakophilins (Pkps) used in this study are listed below. Further Abs for immunofluorescence microscopy and immunoblotting analyses of gel-electrophoretically-separated polypeptides against adhering junction (AJ) proteins and glycoproteins as well as diverse cytoskeletal and nuclear proteins have been described elsewhere (e.g., Barth et al.

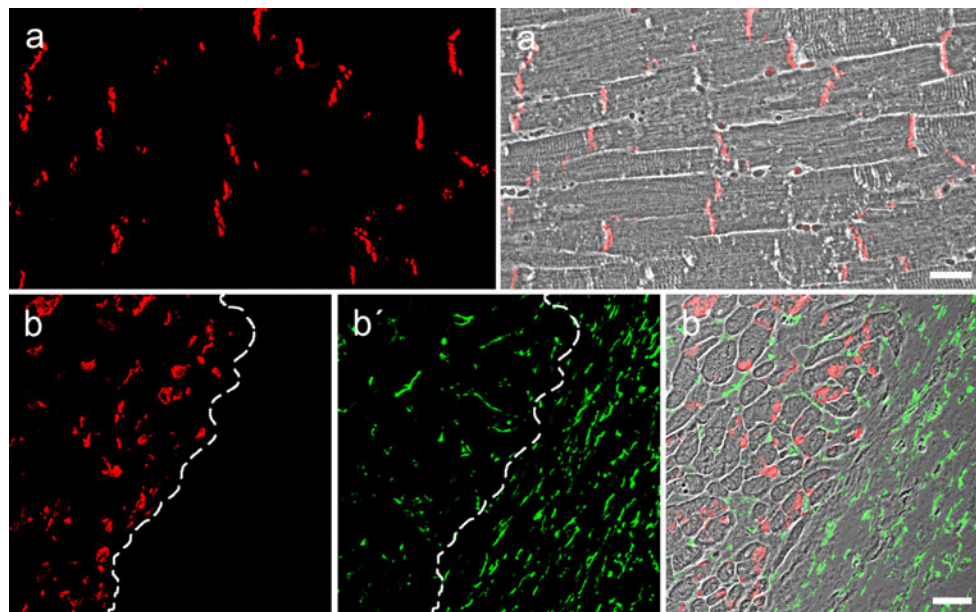


Fig. 2 Immunolocalization of plakophilin-2 (Pkp2) in mammalian heart tissues. Double-label, confocal-laser scanning immunofluorescence microscopy of sections through formaldehyde-fixed and paraffin-embedded human myocardium (**a**, **a'**) or through the border region between a pulmonary valve and the myocardium of an ovine heart (**b–b''**), after antigen-retrieval treatment and reaction with mAbs specific for Pkp2 (red; **a**, **a'**, **b**, **b''**) and polyclonal antibodies (Abs) against the intermediate-sized filament (IF) protein vimentin (green; **b'**, **b''**). Note the positive immunostaining of Pkp2 in the composite junctions (*areae compositae*) of the myocardial intercalated disks (red; **a**, **a'** and in **b** the region on the left hand side of the dashed line) and its absence in the cell–cell contacts between the valvular interstitial

cells (VICs) in the heart valve (**b'**, **b''**; region on the right hand side of the dashed line). Note the widespread occurrence of vimentin-positive VICs, including the myocardial interstitial cells (**b'**; green-stained cells left from the dashed line). The corresponding merged pictures (**a'**, **b''**) are shown on a phase contrast background to allow a clear distinction of myocardial tissue (**b''**; left) and interstitial cells of the valve (**b''**; right). Figures **b–b''** were kindly provided by Dr. Mareike Barth (present address: Institute for Pharmacology and Clinical Pharmacology / Department of Cardiovascular Surgery–Experimental Surgery, University Hospital Duesseldorf, Duesseldorf, Germany). Scale bars 20 μm

2009; Franke and Rickelt 2011; Rickelt et al. 2009, 2011a; Wuchter et al. 2007).

Antigen	Antibody type	Reference
Plakophilin-1	a) mAb, m (PP1-5C2)	Heid et al. 1994
	b) As, gp (GP-PP1)	Heid et al. 1994
Plakophilin-2	a) mAb, m (Pkp2-518)	Rickelt et al. 2010
	b) mAb, m (PP2/62, PP2/86, PP2/150)	Mertens et al. 1996
	c) As, gp (GP-PP2)	Rickelt et al. 2010
Plakophilin-3	a) mAb, m (PKP3-270)	Schmidt et al. 1999
	b) As, gp (GP-PP3-1)	Schmidt et al. 1999

All Abs mentioned were obtained from Progen Biotechnik (Heidelberg). Antigen-bound primary Abs were visualized with secondary Abs coupled to Cy3 (Dianova, Hamburg, Germany) or Alexa 488 (MoBiTec, Goettingen, Germany). 4',6-Diamidino-2-phenylindol (DAPI; Serva, Heidelberg) was applied for staining nuclei (see, e.g., Pieperhoff et al.

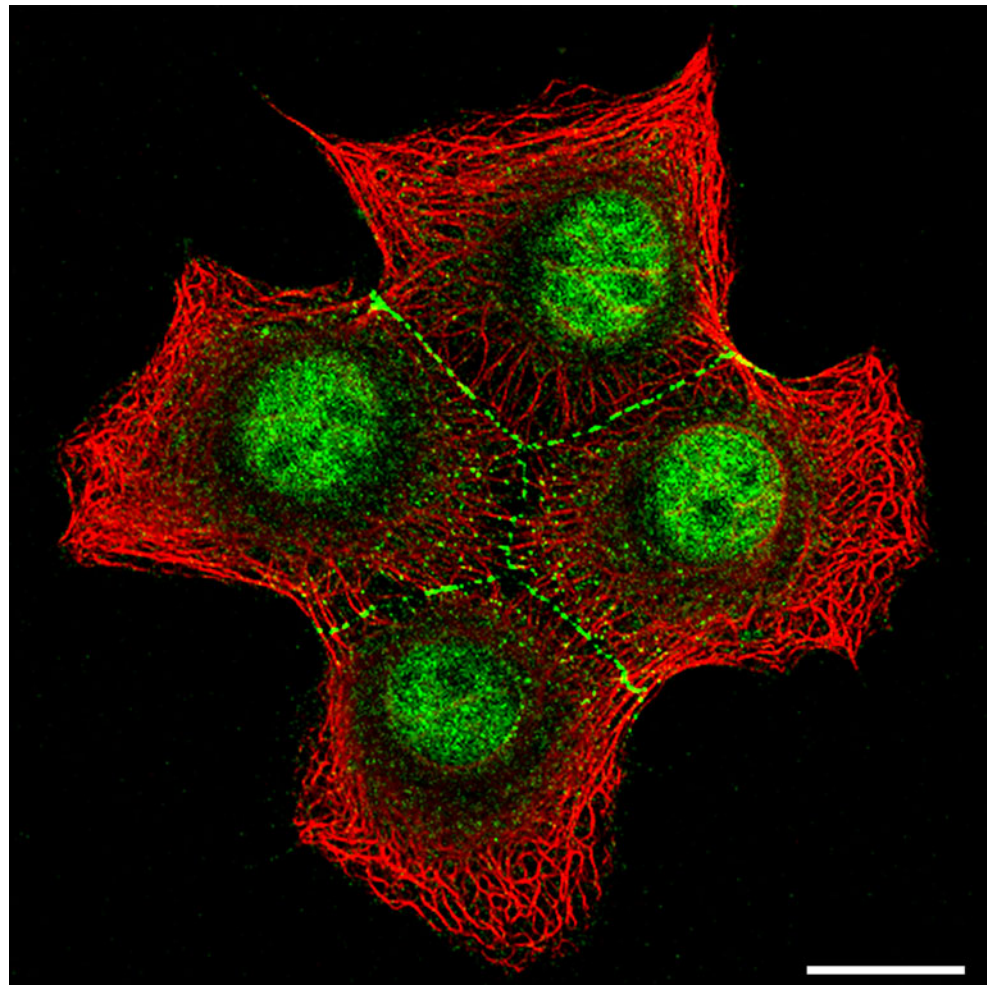
2008). For immunoblot analysis, horseradish peroxidase-conjugated secondary Abs were applied (Dianova).

Immunofluorescence microscopy

Methods for immunofluorescence microscopy were essentially as described for cardiac tissues and cultured cardiomyocytes or interstitial cells in—or from—heart valves (Barth 2011; Barth et al. 2009; Franke et al. 2006; Pieperhoff and Franke 2007; Pieperhoff et al. 2008, 2011; Rickelt 2010; Rickelt et al. 2009, 2010, 2011a).

For the dual—junctional and nuclear—staining of the Pkps on sections through frozen tissues and on monolayers of cultured cells, several procedures were developed (see also previous reports, such as Mertens et al. 1996, 1999; Pieperhoff et al. 2011; Rickelt et al. 2010; Schmidt et al. 1997). Therefore, the cultured cells grown on poly-(L)-lysine-coated coverslips were briefly rinsed with PBS containing 2 mM MgCl₂ and fixed at −20°C in methanol (5 min) and acetone (30 s). The frozen tissue sections were mounted on coverslips, air-dried, and fixed for 10 min in −20°C acetone. The cell or

Fig. 3 Localization of plakophilin-2 (Pkp2) in nuclei and desmosomes of cultured human breast adenocarcinoma-derived cells of line MCF-7. Double-label, confocal-laser scanning immunofluorescence microscopy showing Pkp2 (green) in a small colony of four MCF-7 cells, using polyclonal guinea pig Abs in comparison with the reactions of Abs against keratin IFs (red; murine mAb Lu-5). Note the specific staining of Pkp2 in the typical punctate arrays of desmosomal junctions and the dot-like exo- or endocytotic vesicles associated with desmosomal molecules in the cytoplasm, whereas the cell–cell junction free surface regions are negative. Note in addition the granular fluorescence staining of Pkp2 in the nucleus whereas the nucleoli are negative. Scale bar 20 μm



tissue specimens were rehydrated before incubation with the primary Abs according to one of the following protocols:

- (1) For junctional localization, the specimens were permeabilized in PBS containing 0.2% Triton X-100 for 5 min, rinsed for 5 min with PBS and then treated with the primary Abs for 1 h at room temperature (RT), followed by 3 washes with PBS (5 min each) and incubation with the appropriate secondary Abs (45 min, RT).
- (2) For nuclear staining, the cultured cells or the tissue sections were briefly rinsed with PBS and the primary and secondary Abs were applied for 20 or 30 min each, with three 2–3 min PBS washes between each incubation.
- (3) For optimal dual-localization, the cells were fixed for 5 min in 2% formaldehyde in PBS, freshly made from paraformaldehyde, followed by a brief rinse in distilled water, 5 min quenching in 50 mM NH₄Cl in PBS, another PBS wash and incubation in PBS containing 0.2% Triton X-100 for 5–20 min, before exposure to primary and secondary Abs according to (1).

After the binding of the secondary Abs, the specimens were generally washed again thrice with PBS, briefly rinsed with distilled water and finally dehydrated in absolute ethanol for 1 min. After air-drying, the specimens were mounted with Fluoromount-G (Southern Biotech; obtained through Biozol Diagnostica, Eching, Germany). Finally, immunofluorescence microscopic images were recorded with an Axiophot II photomicroscope (Carl Zeiss, Jena, Germany), equipped with an AxioCam HR (Carl Zeiss). For confocal laser scanning microscopy a Zeiss LSM 510 Meta microscope was used.

For immunolocalizations on paraffin-embedded tissue samples, the sections were deparaffinized and subjected to antigen-retrieval treatment according to standard protocols (see the anthology of Shi et al. 2000; for specific details, see also Rickelt et al. 2010).

Gel electrophoresis and immunoblotting

Tissue samples and cultured cells were analyzed by SDS-PAGE, followed by immunoblotting, as described (Barth 2011; Borrmann et al. 2006; Rickelt 2010; Rickelt et al. 2009, 2010).

Results

Properties of new Pkp2-specific antibodies

As the two major isoforms of Pkp2 (Table 1) based on alternative splicing of the primary gene products and termed

Pkp2a and Pkp2b (Christensen et al. 2010b; Gandjbakhch et al. 2011; Mertens et al. 1996, 1999; Schmidt and Jaeger 2005; Watkins et al. 2009) might differ in their local detectability with immunocytochemical methods and might be located in different positions, I generated antibodies (Abs) of high specificity and affinity allowing reliable Pkp2-reactions on sections through formaldehyde-fixed, paraffin-embedded tissue samples as well as on cultured cells (see also Schmidt et al. 1997). The monoclonal and

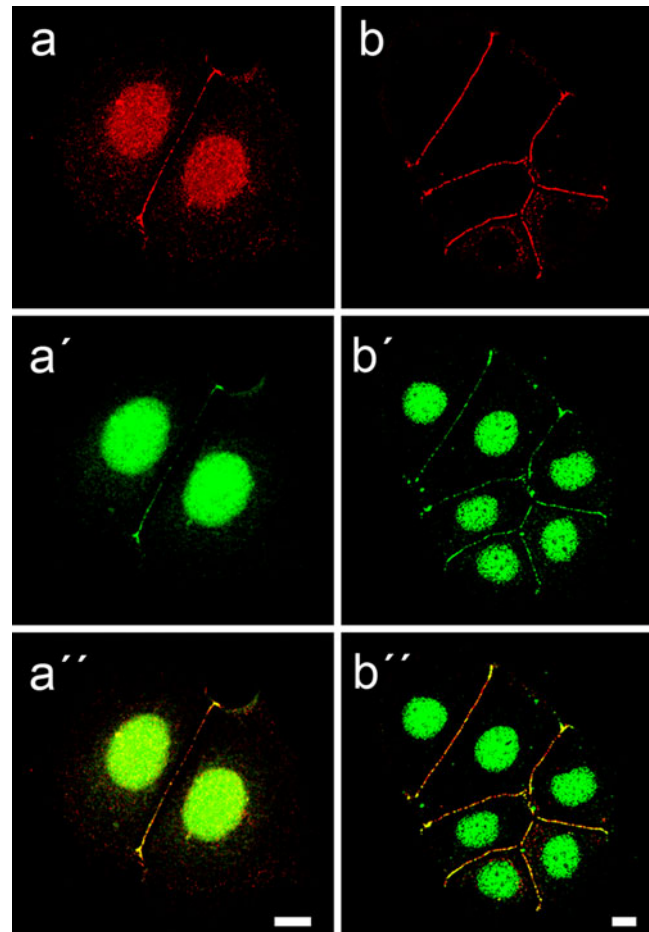


Fig. 4 Specific dual localization of plakophilin-2 (Pkp2) in nuclei and desmosomes of cultured human breast adenocarcinoma-derived MCF-7 cells. **a–a''** Confocal laser-scanning immunofluorescence microscopy of a double-label experiment, comparing the reactions of two Abs against different epitopes of Pkp2 (**a**, red: mAb Pkp2, clone Pkp2-518; **a'**, green: guinea pig Abs of serum GP-PP2) in two adjacent MCF-7 cells. The corresponding merged picture (**a''**) shows the colocalization of both kinds of Pkp2 Abs (yellow merged color) in desmosomes at cell-cell borders and in small granules in the nucleus. **b–b''** Double-label immunolocalization micrographs of small colonies of MCF-7 cells, showing the frequent colocalization of Pkp2 (**b'**, **b''**; green) with the desmosomal plaque protein desmoplakin (**b**, **b''**; red). Note also the intense reaction of Pkp2 in the nucleus, in contrast to the absence of desmoplakin, while both proteins colocalize (**b''**, yellow merged color) in most—but not all—desmosomes in the cell-cell contact regions. Scale bars 10 μ m

polyclonal Abs selected gave essentially similar results with immunoblotting of SDS-PAGE-separated polypeptides of normal and tumor tissue samples, including vertebrate heart tissue and cell cultures (Figs. 1 and 2). Remarkably, all these Pkp2 Abs also revealed the Pkp2 present in the neuroendocrine (“Merkel”) cell half of the hybrid (heterotypic) desmosomes as recently demonstrated (Rickelt et al. 2011a). And, finally, the new Abs reacted rather broadly with the nuclear Pkp2 forms, showing a generally granular nucleoplasmic appearance and leaving the nucleoli negative (Figs. 3, 4, 5 and 7), as well as with cell-cell junctions of diverse desmosome-free cell cultures (Figs. 4, 6 and 7).

Pkp2 reactions on non-epithelial, non-cardiomyocytic cells

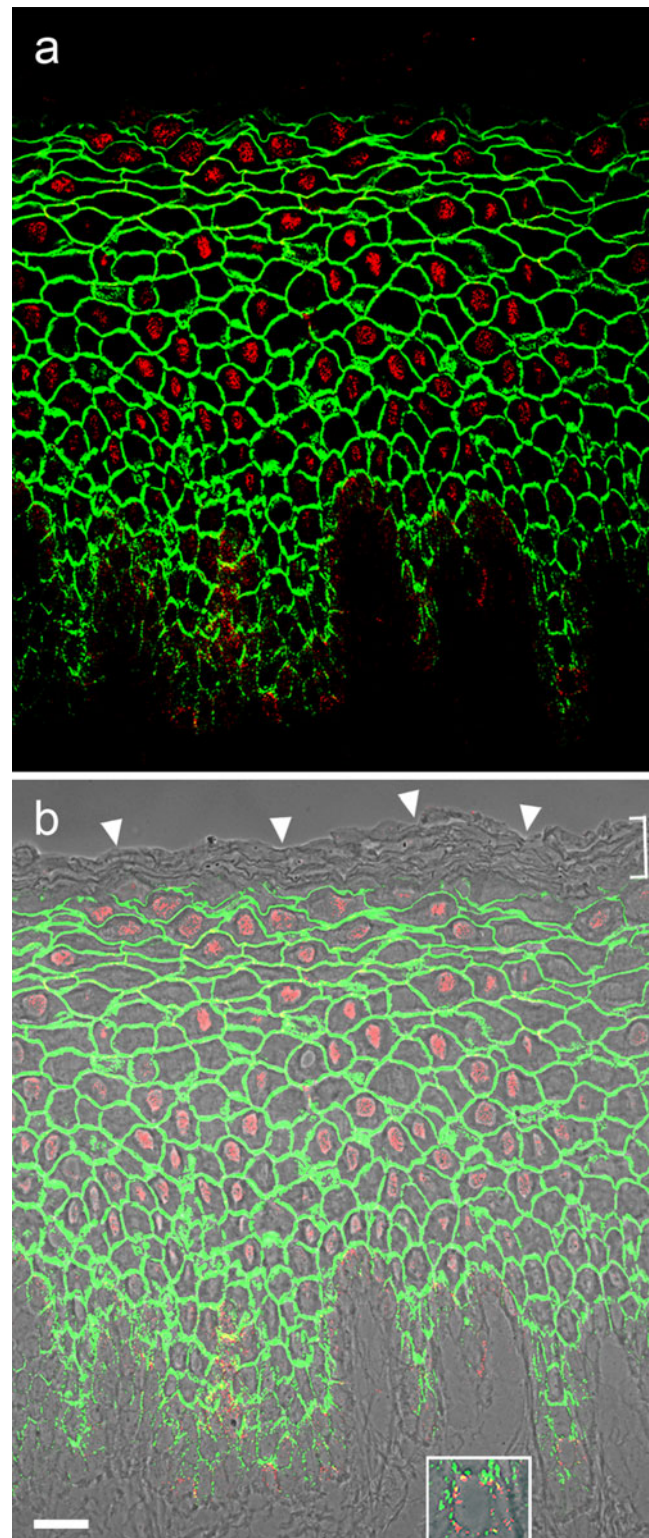
The Pkp2-positive reactions also revealed nuclear structures in cultured cells known to be totally devoid of desmosomes and any other adherens junctions (AJs; e.g., Fig. 7c–c’). Furthermore, using unmasking immunostaining conditions (e.g., procedure 3 of “Materials and methods”) it was possible to detect and localize two major Pkp2 forms simultaneously, one in small nucleoplasmic granules (Fig. 7a, b) and the other in non-desmosomal AJs of the *puncta adhaerentia* or *fasciae adhaerentes* types (Figs. 6a–c and 7a, b). Simultaneously, i.e. in the same cells and under the same immunocytochemical conditions, it could also be demonstrated that such non-desmosomal cell–cell AJs immunostained for Pkp2 were also positive for N-Cadherin, α - and β -catenin (Fig. 6a–c) as well as for cadherin-11 and for the *armadillo* proteins plakoglobin, p120 and p0071 (not shown here; see also Rickelt et al. 2009).

At present, it cannot be explained why certain cell lines derived from different species and tissues synthesize remarkable amounts of Pkp2 (Fig. 8), whereas others, including some directly related to each other, are negative (compare, e.g., lines WI38 and WI38VA13 in Fig. 8; see also Rickelt et al. 2009).

Fig. 5 Nuclear localization of plakopilin-2 (Pkp2) in fetal porcine snout epithelium. **a, b** Immunofluorescence microscopy showing the localization of polyclonal guinea pig Abs specific for Pkp2 (red) on cryostat sections through fetal porcine snout epithelium in comparison with the desmosomal plaque protein desmoplakin (green). Under these immunostaining conditions used here the intense and specific nuclear localization of Pkp2 (red) contrasts with the desmoplakin immunostaining restricted to the desmosomes of all keratinocytes. Note also the absence of both the uppermost layers of the *stratum corneum* (**b**, bracket; the tissue surface is denoted by the arrowheads). The picture of (**b**) is presented on a phase contrast background. Note in addition that Pkp2 is—in addition to the nuclei—also specifically located in distinct “dot-like” structures in the basal cell layer, representing one half of the heterotypic desmosomes connecting the keratinocytes and the neuroendocrine “Merkel cells” (*insert in b* shows a magnification of one of the Merkel cells in the basal layer in the *right lower corner*). Scale bar 20 μ m

Cardiac myxomata

When the more sensitive new Abs were examined on diverse non-epithelium-derived cell culture types and tissues, it soon became obvious that the cells of many non-carcinomatous



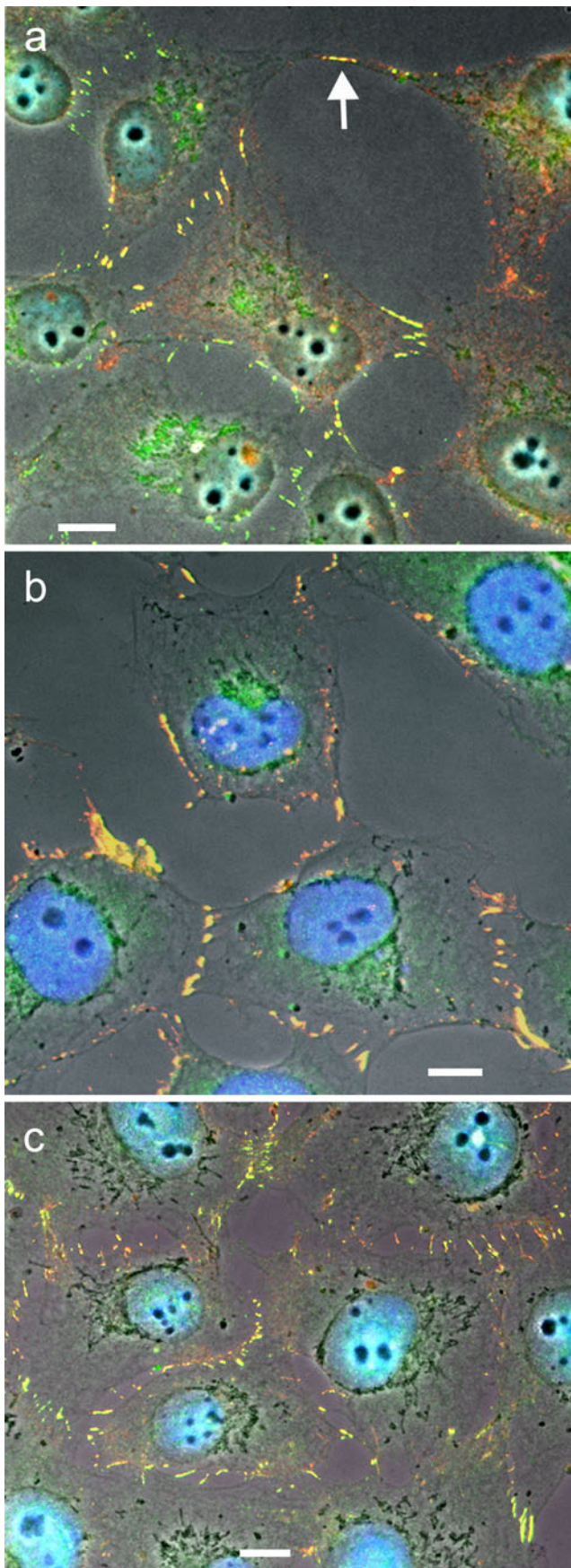


Fig. 6 Identification and localization of plakophilin-2 (Pkp2) in adherens junctions (AJs) of highly proliferating cultured human fibroblasts. Double-label immunofluorescence microscopy images of SV40-transformed human fibroblasts (line SV80), after reaction with Abs to Pkp2 (**a**, red; murine mAbs, or **b** and **c**, green; guinea pig Abs), in comparison with constitutive AJ proteins such as the transmembrane glycoprotein N-cadherin (**a**, green; polyclonal rabbit Abs) and the (red-labeled) murine mAbs specific for the cytoplasmic AJ plaque proteins α - (**b**) and β -catenin (**c**). Note that both kinds of Pkp2 Abs clearly colocalize with the AJ proteins (yellow merged color) not only when grown at low density (**a**, **b**) but also near confluency (**c**). In addition, such colocalization can also be seen in small cell-cell junctions connecting slender cell processes (see, e.g., arrow in **a**). DAPI staining (blue) was used to visualize the nuclei. Scale bars 10 μ m

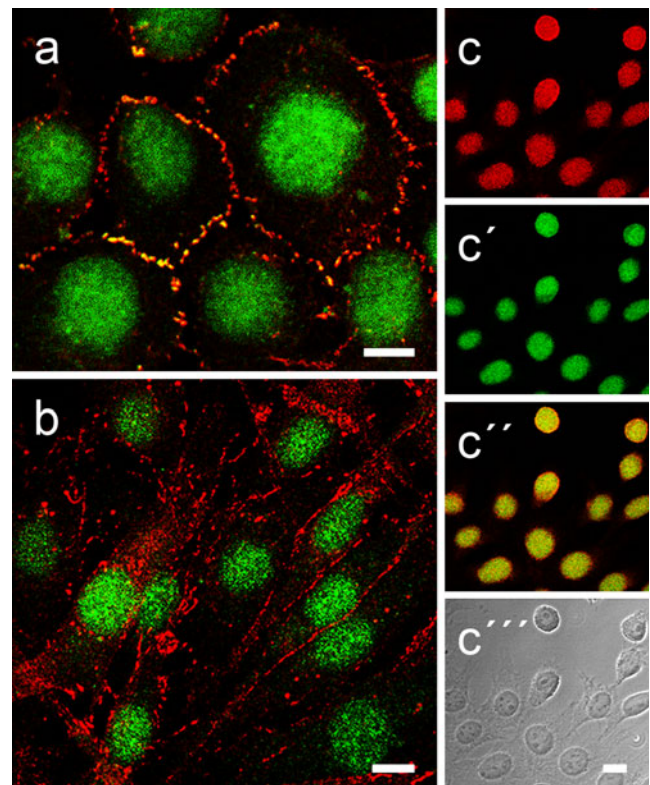


Fig. 7 Differential localization of plakophilin-2 (Pkp2) in mammalian fibroblasts. Double-label, laser-scanning immunofluorescence microscopy of cultured transformed human fibroblasts of line SV80 (**a**), bovine dermal fibroblasts of line B1 (**b**) and mouse fibroblasts of strain L929 after formaldehyde fixation and detergent-treatment (for details, see “Materials and methods”). Here, the immunolocalization of polyclonal guinea pig Abs specific for Pkp2 (**a**, **b**, **c'**, **c''**; green) and the adherens junction (AJ) proteins N-cadherin (**a**, **b**; red) and β -catenin (**c**, **c''**; red) is shown. Under these conditions, both the specific staining of junctions at the cell–cell boundaries and the intense nuclear staining are seen. Note, however, that in the transformed fibroblasts (**a**), Pkp2 occurs in the N-cadherin-positive AJs (yellow merged color) whereas AJs of untransformed B1 fibroblasts lack Pkp2. In all fibroblasts, Pkp2 is also abundant in the nucleus (**a**–**c**) in comparable amounts as β -catenin as shown in the “junction-lacking” L929 fibroblasts (**c**–**c''**). The corresponding merged color picture (**c'**) and the phase contrast background (**c'''**) images are presented in addition in order to see the cytoplasms of these cells. Scale bars 10 μ m

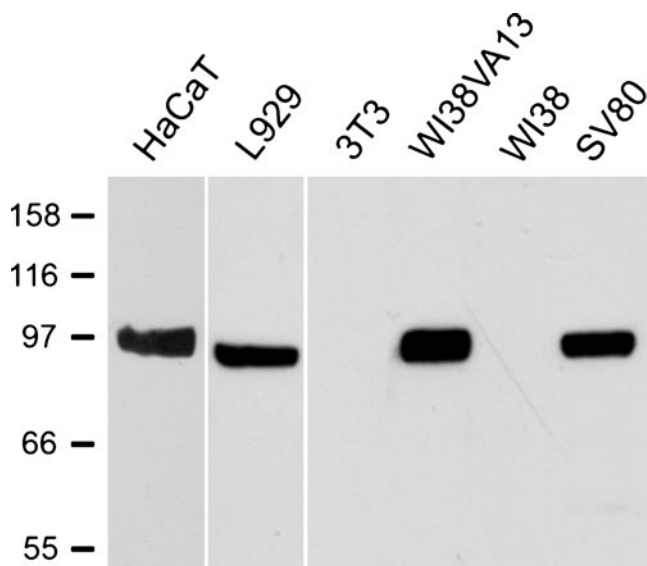
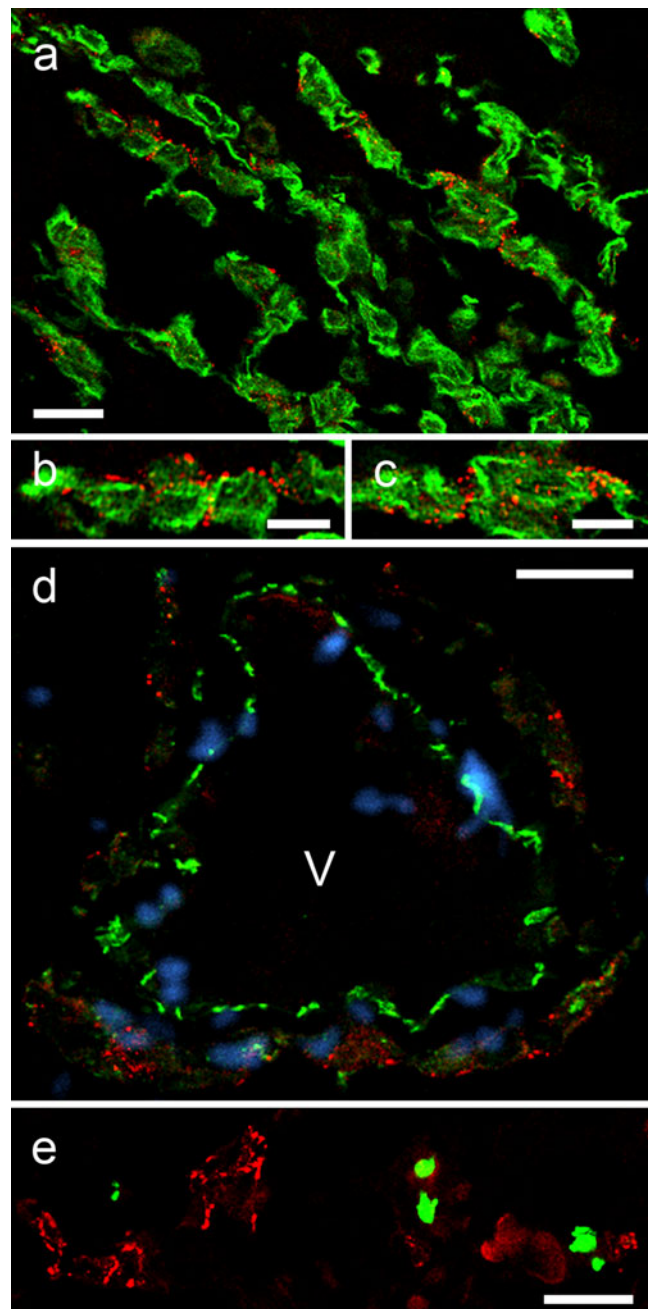


Fig. 8 Immunoblot specificity of plakophilin-2 (Pkp2) on polypeptides of total protein lysates of murine and human fibroblastoid cells. Immunoblot reaction of murine mAbs specific for Pkp2 on SDS-PAGE-separated polypeptides presents in lysates of permanently growing human keratinocytes of line *HaCaT* (lane 1), murine fibroblasts of lines *L929* (lane 2) and *3T3* (lane 3), SV40-virus transformed (line *WI38VA13*; lane 4) and non-transformed (lane 5) human diploid lung fibroblasts of line *WI38* and SV40-virus transformed human fibroblasts of line *SV80* (lane 6). Note that the Pkp2-specific polypeptide band of ca. 97 kDa appears not only in lysates of *HaCaT* keratinocytes and transformed fibroblasts (*WI38VA13* and *SV80*) but also in the “junction-lacking” *L929* fibroblasts. Positions of polypeptide molecular weights are indicated on the left and correspond to 158, 116, 97, 66 and 55 kDa (from top to bottom)

tumor cells were connected by non-desmosomal cell–cell junctions containing cadherin-11, N-cadherin, α - and β -catenin and plakoglobin as well as proteins p120 and p0071 (e.g., Rickelt et al. 2009, 2010). Such cells included in particular one group of cardiac tumors, the “cardiac myxomata“, the nucleus-containing cell bodies of which were for the most part distant from each other and separated by extracellular matrix

Fig. 9 Localization of plakophilin-2 (Pkp2) in adherens junctions (AJs) of human cardiac myxomata. Laser-scanning, double-label immunofluorescence microscopy of sections through formaldehyde-fixed and paraffin-embedded human myxomata, treated for antigen-retrieval and double-immunostained with mAbs to Pkp2 (red), in comparison with green-labeled polyclonal Abs decorating IF bundles of the vimentin-type (a–c), the vascular endothelial transmembrane glycoprotein VE-cadherin (d) and the cell proliferation marker protein Ki67 (e). Note the extensive localization of Pkp2 in the AJs connecting myxoma cell processes, which also contain vimentin IFs (a–c). b, c Higher magnification micrographs of the region shown in (a), demonstrating that Pkp2-positive *puncta adhaerentia* are often clustered and appear as “beaded chains”. d Note here that the AJs connecting the endothelial cells of vessels (V), which are recognized by their intense VE-cadherin staining, are negative for Pkp2. Moreover, in myxomata, only a few cells undergo cell division as seen by immunostaining with Ki67 as proliferation marker (e). DAPI staining (blue) was used to visualize the nuclei in (d). V vessel lumen. Scale bars 10 μ m (b, c), 20 μ m (a, d, e)

structures (e.g., Fig. 9a), as is typical for such tumors (for references, see McAllister and Fenoglio 1978; Sheppard 2011; Travis et al. 2004). These frequent and mostly rather long cell processes often showed cell–cell contacts with an AJ morphology, which by electron microscopy (e.g., Rickelt et al. 2010) as well as in their molecular composition were negative for all desmosome-type components but strongly positive for Pkp2 colocalizing with the various non-desmosomal junction molecules mentioned (Fig. 9a–d; b and c show partial magnifications of AJ clusters at small contact sites between two tumor cells). As these cells were all positive for the IF protein



vimentin and negative for keratins, desmin and the typical epithelial junction marker proteins, desmoglein Dsg2, desmocollin Dsc2 and desmoplakin and also for α -cardiac actin and the myocardial variants of troponin and myosin (for applications of myocardial markers in tumor diagnoses, see, e.g., Moll et al. 2006), any relationship to carcinomatous or myocardium-derived tumors appeared to be excluded. Moreover, as indicated by the absence of VE-cadherin reactions (e.g., Fig. 9d) and factor VIII-related antigen (not shown), any derivation of these tumor cells from vascular endothelial cells was also excluded (for the rather modest positivity with the nuclear proliferation index protein Ki57 see Fig. 9e).

Discussion

The special importance of plakophilin-2 (Pkp2) in the formation and architectonic organization of the desmosomal junctions as well as of other, more complex, junctions such as the composite junctions (CJs) of the myocardial intercalated disks has first been directly demonstrated by the successive cumulative transfection experiments of Koeser et al. (2003) and most impressively in the gene knock-out experiments of Grossmann et al. (2004; for special details, see also Grossmann 2005). In addition, immunoelectron microscopic localization studies have shown that at least a portion of Pkp2 is positioned very close to the desmosomal or CJ membrane (Borrmann 2000; Franke et al. 2006; Mertens et al. 1996; for the “molecular location maps” of other Pkps, see also North et al. 1999; Bass-Zubek et al. 2009). Certainly, the most eye-opening experimental results in this respect were the observations of Grossmann et al. (2004) that, in the forming embryonic heart, the normal architectonic order of the assembly of the cardiac structural elements was lost in the absence of Pkp2, so that a large portion of desmoplakin, the major plaque protein,

appeared in non-ordered aggregates dispersed over the cytoplasm far away from the plasma membrane and that this loss of order then led to the disruption of the myocardial structure and cardiac death.

Another relevant biochemical result demonstrating the importance of Pkp2 in the cardiomyocyte CJs was the finding that the myocardium-specific plaque protein α -T-catenin binds to Pkp2 and that this interaction is essential for both the correct topology and function of these proteins in the CJs as well as for the functions of connexin-43 (Cx43) in the adjacent gap junctions (Goossens et al. 2007; Li et al. 2011; for functional interdependence of Pkp2 and Cx43, see also Fidler et al. 2008; Oxford et al. 2007a, b; Sato et al. 2009).

Striking experimental evidence that Pkp2 is of special importance in the formation and maintenance of the CJs of the forming intercalated disks in peri- and postnatal rodents is also provided by the observations with cultured cardiomyocytes that direct interference with the freshly formed and just forming junction structures by siRNAs using Pkp-mRNA sequences results in the splitting and cleavage of these junctions along their “midplane”, followed by the dissociation of the then uncoupled cardiomyocytes and the dispersion of the junctional components over the cytoplasm (Fidler et al. 2008; Hall et al. 2009; Oxford et al. 2007a, b; Pieperhoff et al. 2008). In view of these experimental findings, it now appears less surprising that Pkp2 has also been identified as the by far most frequent and sensitive mutation target resulting in situations of cardiac damage and even sudden death of the arrhythmogenic ventricular cardiomyopathy/dysplasia (ARVC/D) type (Table 2; for references, see also Calkins 2006, 2011; Cox et al. 2010; Delmar and McKenna 2010; Li and Radice 2010; MacRae et al. 2006; Marcus et al. 2007; Pieperhoff et al. 2010; Sen-Chowdhry et al. 2007).

By contrast, our insight into the nuclear Pkp2 complexes and structures formed and the functions served by nuclear

Table 2 Cardiological publications presenting examples for the important role of plakophilin-2 in the development of arrhythmogenic ventricular cardiomyopathies (ARVC), including “sudden death”

For recent reviews and anthologies, see also: Azaouagh et al. 2011; Delmar and McKenna 2010; Herren et al. 2009; Marcus et al. 2007; Pieperhoff et al. 2010.

Gerull et al. 2004	Lahtinen et al. 2008	Fressart et al. 2010
Antoniades et al. 2006	Ram and van Wagoner 2008	van der Zwaag et al. 2010
Awad et al. 2006	Tandri et al. 2008	Xu et al. 2010
Basso et al. 2006	Yu et al. 2008	Aneq et al. 2011
Calkins 2006	Bhuiyan et al. 2009	Basso 2011
Dalal et al. 2006	den Haan et al. 2009	Calkins 2011
Kannankeril et al. 2006	Hall et al. 2009	Gandjbakhch et al. 2011
Nagaoka et al. 2006	Qiu et al. 2009	Lahtinen et al. 2011
Syrris et al. 2006	Watkins et al. 2009	Li et al. 2011
Tsatsopoulou et al. 2006	Wu et al. 2009	Lombardi and Marian 2011
van Tintelen et al. 2006	Barahona-Dussault et al. 2010	Paul et al. 2011
Otterspoor et al. 2007	Bauce et al. 2010	Rickelt et al. 2011b
Sen-Chowdhry et al. 2007	Christensen et al. 2010a	van Tintelen 2011
Fidler et al. 2008	Christensen et al. 2010b	
Joshi-Mukherjee et al. 2008	Cox et al. 2010	

Pkp2 is still very limited. So far, only an RNA polymerase III complex obviously of regulatory importance has been isolated and characterized (Mertens et al. 2001) and Mueller et al. (2003) have reported nuclear Pkp2 phosphorylation in a specific way, followed by complexing with the 14-3-3 protein. Obviously, the nucleoplasm contains several further Pkp2-containing complexes (Mertens et al. 2001) and the intense and specific binding of the antibodies (Abs) to nucleoplasmic structures (see, e.g., Figs. 3, 4, 5 and 7) may now help in isolating such particles and in the elucidation of the nature, reactions and functions of the various nuclear Pkp2 forms. Here, it will be also of experimental help to use junction-free cell lines that only contain nuclear Pkp2 forms such as the murine L929 fibroblastoid cells (Fig. 7c–c’'). And the armamentarium of the new Pkp2 Abs will also help to decide which isoforms or posttranslational modifications exist in exchange equilibria between nucleoplasm, cytoplasm and junctional plaques and what principles regulate the differential topology of the different Pkp molecules.

And, finally, the set of novel Pkp2 Abs presented here should also contribute to progress in physiologies, pathology and regenerative medicine. For example, the constitutive presence of Pkp2 in non-desmosomal adherens junctions (AJs) connecting certain kinds of mesenchymal or mesenchymally-derived cells of embryonal and fetal mammalian tissues, as well as the demonstrated rapid and stable integration of new Pkp2 molecules in the non-desmosomal junctions connecting mesenchymal or mesenchymally-derived cells, seem to allow the growing and proliferation of such cells in culture, a situation that can quickly return to Pkp2-devoid AJs upon reimplantation of these cells into the natural habitat (for details, see Barth 2011; Barth et al. 2009, 2011). It is also safe to predict that Pkp2 will soon play an increasingly important role in diagnostic pathology, in particular in the diagnoses of non-epithelial tumors, including cardiac and non-cardiac myxomata.

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