A novel *MYH6* Arg568Cys variant not seen in 246 controls was detected.[20] DNA from additional relatives was not available.

Pedigree F

The proband, a non-Hispanic white woman, presented with paroxysmal nocturnal dyspnea, cardiomegaly on chest x-ray, and heart failure. PPCM was diagnosed 1 week postpartum (Table 3). A nonsynonymous *TNNT2* Arg159Gln variant was identified that occurred at a conserved site and was absent in 253 controls.[15] Functional studies demonstrated decreased calcium sensitivity, which indicated that this mutation was likely to be disease causing.[21]

Discussion

From our database designed for DCM genetic studies, we present cases of DCM onset occurring in pregnancy or the immediate postpartum period and their molecular genetic rare variant data, suggesting genetic causation. Among the 4110 women in the 520 pedigrees analyzed in our FDC cohort, we identified 45 PPCM or PACM cases. In 6 of the 19 cases for which resequencing data were available, mutations were identified in genes that have been shown previously to be associated with DCM. PPCM was diagnosed in 5 (pedigrees B, C, D, E, F) and PACM in 1 (pedigree A). To our knowledge, this is the first cohort of PPCM/PACM cases with sequencing data from genes that are relevant for genetic DCM.

Identifying the cause or causes of PPCM/PACM has been elusive, and no compelling data have previously supported any 1 central hypothesis.[23] Numerous causes have been proposed, including autoimmune processes, myocarditis, abnormal hemodynamic responses to pregnancy, and selenium deficiency.[23–26] Other proposed risk factors include maternal age >30 years old, twinning, hypertension, preeclampsia, and tocolytic therapy.[1,4,23,24,27] Although African descent has also been proposed as a risk factor, this association may be confounded by socioeconomic status in some populations.[27] Genetic cause has also been suggested in some studies, as noted above.

Because our ≥15-year study has been devoted primarily to identifying patients with DCM who may have familial disease, it is not surprising that some of the participants whom we identified, otherwise meeting criteria established for the diagnosis of PPCM or PACM, had familial disease. Previous reports[6–11] of individual familial cases are at variance, as is this report, with several series of PPCM cases that have reported sporadic disease. For example, none of the PPCM subjects reported in 2 key studies[2,4] had positive family histories. One possible explanation for this difference is that the pathogenic basis of the PPCM in those reports[2,4] may have been different (and nonhereditary) from that seen in our series of patients. Another possibility is that evidence of familial disease was not detected because neither of the prior studies[2,4] specified that a family history had been obtained. The fact that a careful, prospective 3- to 4-generation family history is essential to detect familial disease has been well established for DCM.[5,28,29] It has also been well established that the family history is insensitive to detect familial DCM compared with clinical screening (history, examination, ECG, and echocardiography) of closely related family members. This latter fact has led to the recent guideline recommending clinical screening for first-degree relatives of all patients newly diagnosed with DCM.[29] We suggest that this clinical guideline recommendation also be considered for cases of suspected PPCM/PACM and that a 3- to 4-generation family history (and consideration of clinical screening of first-degree family members) be integrated into all ongoing PPCM/PACM research study designs.

Some have suggested that the PPCM diagnosis should be distinguished from FDC.[30] However, we suggest the alternative possibility, that genetic DCM may underlie a significant proportion of PPCM cases, regardless of a positive family history. This point is illustrated by pedigrees E and F, in which both probands met PPCM criteria and were the only known affected individuals in their family yet carried possibly or likely disease-causing DCM mutations, respectively. Prospective registries assessing the presence of DCM clinical findings and mutations in first-degree relatives of PPCM/PACM probands will be necessary to further evaluate this possibility.

Limitations

Because of the nature of our study design, we were unable to obtain all cardiovascular data from all subjects with a history of PPCM/PACM. However, we restricted our cardiovascular and clinical genetics assignments to those for whom requisite clinical data were available. The previously published data or the data presented herein to prove causation varied for each mutation. For mutations in 2 genes, *TNNT2* and *PSEN2*, the previously published functional data[19,21] in concert with the segregation of the variant with DCM in multiple family members supported their role as highly likely disease-causing variants. For other mutations, segregation of DCM with the variant in *SCN5A* in multiple other family members (pedigree C; Figure) or the prior report of the *MYBPC3* variant in association with DCM[22] supports their likely disease-causing roles. Mutations in 2 genes (*MYH6*, *MYH7*) had no functional or segregation data available. However, the *MYH7* gene encodes the key sarcomeric protein β-myosin heavy chain, which has had multiple previous mutations reported in association with DCM,[12,15,31–33] and the *MYH7* mutation identified as pedigree B met usual criteria (a rare, nonsynonymous variant) to be considered as possibly causing disease. The *MYH6* gene encodes α-myosin heavy chain, another sarcomeric gene with variants that have been reported previously in association with DCM.[20,34] Although the *MYH6* variant we identified in pedigree E was also nonsynonymous and rare, because the role of *MYH6* variants in DCM is overall less well established, the evidence that this variant is possibly disease causing is not as strong as that of the *MYH7* variant. We also note that our resequencing data were limited to those genes known to cause DCM in male and female subjects of all ages without regard to the PPCM/PACM diagnosis, and therefore it is possible that genes more relevant to cardiac function during pregnancy or the immediate postpartum period were missed; resequencing studies of additional genes will be required to assess this possibility. We

are unable to specify the degree to which our cases are representative for PPCM/PACM because our study was not designed specifically to identify PPCM/PACM cases per se, and the PPCM/PACM case series have not presented systematically obtained family history and pedigree data. However, our cases met usual criteria for PPCM or PACM and are relevant to establish that a genetic cause may underlie some proportion of PPCM/PACM.

Implications for Clinicians

This report has implications for clinicians caring for PPCM and PACM patients, as well as their families. For PPCM/PACM patients, the approach taken should be the same as that recommended for a new IDC diagnosis,[29] which includes consideration of the possibility of FDC and of genetic cause. A genetic evaluation including family history, clinical screening, and genetic counseling and testing should be conducted for the proband and for first-degree relatives.[29] Although PPCM and PACM are rare, these conditions may occur more frequently among relatives of patients with IDC, and therefore reproductive risk counseling about PPCM/PACM is appropriate for female first-degree relatives of probands with IDC in the context of a genetic cardiomyopathy evaluation.[29]

Conclusion

Mutations associated with DCM were present in some subjects meeting formal criteria for PPCM/PACM, suggesting that a proportion of PPCM/PACM may result from a genetic cause and even in the absence of a disease-positive family history. These findings have implications for further research and may be of critical importance in the management of women with PPCM and their families.

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Disclosures

None.

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CLINICAL PERSPECTIVE

The term *peripartum cardiomyopathy* (PPCM) describes dilated cardiomyopathy (DCM) without known cause that occurs during the last month of pregnancy to 5 months postpartum. A related term, *pregnancy-associated cardiomyopathy* (PACM), refers to DCM onset earlier in pregnancy. Despite multiple studies focused on inflammatory, immunologic, and environmental causes, no unifying hypothesis has been proven. An alternative hypothesis is that PPCM and PACM result from a genetic cause. In an effort to identify preliminary support for this hypothesis, a systematic search of a large database, collected for family-based genetic DCM studies over the past 15 years, was undertaken for cases associated with pregnancy and the postpartum period. When cases were identified, available clinical and molecular genetic data were analyzed. Of the 4110 women from 520 pedigrees in the Familial Dilated Cardiomyopathy Research Project database, 45 cases of PPCM/PACM were identified, 23 with familial clustering, of which 19 had been resequenced for known DCM genes. Six of these 19 carried mutations in genes shown previously to be associated with DCM. These data indicate that PPCM/PACM may have a genetic basis in some cases. Thus, we recommend that clinicians caring for PPCM/PACM patients be aware that PPCM/PACM may have a genetic basis and that guidelines for evaluation of genetic cardiomyopathy be followed. Specifically, this includes clinical screening (family history, medical history, examination, ECG, echocardiography) of the patient and her first-degree relatives. Genetic counseling, including reproductive risk counseling about PPCM/PACM, is recommended, in addition to consideration of genetic testing.

Peripartum Cardiomyopathy as a Part of Familial Dilated Cardiomyopathy

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Background—Anecdotal cases of familial clustering of peripartum cardiomyopathy (PPCM) and familial occurrences of PPCM and idiopathic dilated cardiomyopathy (DCM) together have been observed, suggesting that genetic factors play a role in the pathogenesis of PPCM. We hypothesized that some cases of PPCM are part of the spectrum of familial DCM, presenting in the peripartum period.

Methods and Results—We reviewed our database of 90 DCM families, focusing specifically on the presence of PPCM patients. Then, in a reverse approach, we reviewed 10 PPCM patients seen in our clinic since the early 1990s and performed cardiological screening of the first-degree relatives of 3 PPCM patients who did not show a full recovery. Finally, we analyzed the genes known to be most commonly involved in DCM in the PPCM patients. We identified a substantial number (5 of 90, 6%) of DCM families with PPCM patients. Second, cardiological screening of first-degree relatives of 3 PPCM patients who did not show full recovery revealed undiagnosed DCM in all 3 families. Finally, genetic analyses revealed a mutation (c.149A>G, p.Gln50Arg) in the gene encoding cardiac troponin C (*TNNC1*) segregating with disease in a DCM family with a member with PPCM, supporting the genetic nature of disease in this case.

Conclusions—Our findings strongly suggest that a subset of PPCM is an initial manifestation of familial DCM. This may have important implications for cardiological screening in such families. (***Circulation.* 2010;121:2169-2175.)**

Key Words: cardiomyopathy ■ genetics ■ pregnancy

Peripartum cardiomyopathy (PPCM) is a rare, life-threatening cardiomyopathy that affects women late in pregnancy or in the early puerperium. Diagnostic criteria for PPCM are (1) development of cardiac failure in the last month of pregnancy or within 5 months after delivery, (2) left ventricular systolic dysfunction (left ventricular ejection fraction <45%), (3) no identifiable cause for cardiac failure, and (4) no recognized heart disease before the last month of pregnancy.[1–3]

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Clinical Perspective on p 2175

A number of risk factors for PPCM have been identified, including advanced maternal age, multiparity, and eclampsia. In addition, several possible underlying pathological processes have been identified such as myocarditis, abnormal autoimmune responses, apoptosis, and impaired cardiac microvasculature.[4,5] Recent data have shown that unbalanced peripartum/postpartum oxidative stress is linked to proteolytic cleavage of prolactin into a potent antiangiogenic, proapoptotic, and proinflammatory factor, but the cause of PPCM is still not fully understood.[6]

The position statement from the European Society of Cardiology on the classification of cardiomyopathies classifies PPCM as a nonfamilial, nongenetic form of dilated cardiomyopathy associated with pregnancy.[7] However, anecdotal cases with familial clustering of PPCM, as well as familial occurrences of PPCM and idiopathic dilated cardiomyopathy (DCM), have been reported, suggesting that genetic factors play a role in the pathogenesis of PPCM.[8–15] Genetic analysis is not usually performed in PPCM, and so far, no mutations in genes related to hereditary cardiomyopathies have been reported for it.

PPCM probably develops as a result of a complex interaction of pregnancy-associated factors and genetic factors. In other words, against a background of genetic susceptibility, factors associated with pregnancy could lead to PPCM. In this study, we focused on the genetic/familial component of PPCM. We hypothesized that a subset of PPCM cases is part of the spectrum of familial DCM, presenting in the peripar-

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tum period. We followed a 2-tier approach to test this hypothesis: We examined a large Dutch cohort of proven familial DCM cases and reported on the number of PPCM patients found in this cohort, and in the reverse order, we reviewed all the PPCM patients seen in our clinic since the early 1990s and performed cardiological screening of the first-degree relatives of PPCM patients who did not show full recovery. In addition, we analyzed the genes known to be most commonly involved in DCM in these cases. In 1 family, we performed more extensive genetic screening because the index patient was very young at the onset of DCM.

Methods

Clinical Evaluation

PPCM Patients in Families With DCM

Patients with idiopathic DCM or other possible hereditary cardiac disorders are routinely evaluated at the cardiogenetics outpatient clinic of the University Medical Centre Groningen. Our routine procedures include recording the clinical characteristics of the index patient, constructing a pedigree, and inquiring about the family history. Letters are provided for family members at risk to invite them to the outpatient clinic for presymptomatic ("cascade") screening.[16,17]

DCM is diagnosed when a patient has both a reduced systolic function of the left ventricle (left ventricular ejection fraction <0.45) and dilation of the left ventricle (left ventricular end-diastolic dimension >117% of the predicted value corrected for body surface area and age) and only after identifiable causes like severe hypertension, coronary artery disease, and systemic diseases have been excluded.[18,19] If only one of these criteria is fulfilled, the case is labeled mild DCM. Familial DCM is diagnosed if there are ≥2 affected family members or if a first-degree relative of a DCM patient died suddenly before 35 years of age.[19] We reviewed our database of DCM families, focusing specifically on the presence of PPCM patients in these families and using the diagnostic criteria for PPCM mentioned earlier.[1–3] The local institutional review committee approved the study, and all participants gave informed consent.

PPCM Patients and Cardiological Screening of Their Family Members

We reviewed all the PPCM cases collected since the early 1990s by the Department of Cardiology, University Medical Centre Groningen. The available data on the patients' clinical characteristics were carefully evaluated. If a patient had not fully recovered from PPCM within 1 year (normalization of left ventricular function and dimensions), she was invited to the cardiogenetics outpatient clinic to be informed about the possibility of family screening for DCM and DNA analysis. Patients were given family letters to give to their relatives. Those relatives who responded and who, after undergoing genetic counseling, agreed to cardiological screening were subjected to a 12-lead ECG, an echocardiogram, and magnetic resonance imaging (if indicated). The local institutional review committee approved the study, and all the participants gave their informed consent.

Genetic Analysis

Genomic DNA was isolated from blood samples obtained from the index patients of the DCM families with a case of PPCM or from the PPCM patients. DNA samples from 300 ethnically matched control alleles were used as control. DNA analysis was performed for the following DCM-related genes: lamin A/C (*LMNA*), cardiac troponin T (*TNNT2*), and β-myosin heavy chain (*MYH7*). We used genetic techniques (denaturing high-performance liquid chromatography, denaturing gradient gel electrophoresis, and direct sequencing) to screen the protein-coding regions of the exons, as well as the adjacent intronic regions essential for splicing. To detect large deletions or duplications of ≥1 exons of *LMNA*, we used the multiplex ligation-dependent probe amplification test (MRC-Holland, Amsterdam, the Netherlands).[20]

In family 1b, we performed more extensive DNA analysis because the index patient developed DCM at a very young age (before 2 years of age). In this family, we analyzed almost all the DCM-related genes available in diagnostics (ie, *ACTC1* [cardiac α-actin], *CSRP3* [muscle LIM protein], *DES* [desmin], *LMNA*, *MYBPC3* [myosin-binding protein C], *MYH7*, *TNNC1* [cardiac troponin C], *TNNI3* [cardiac troponin I], *TNNT2*, *TPM1* [α-tropomyosin]). Details of all these analyses are available on request.

Results

PPCM Patients in Families With DCM

Ninety families with idiopathic DCM were available for investigation, and we found 5 families (5 of 90, 6%) with at least 1 case of PPCM. Detailed clinical data of these PPCM patients and their affected family members (families 1a through 1e) are given in Table 1, and their pedigrees are shown in Figure 1. In family 1d, 2 members with PPCM were identified. In family 1b, we identified 1 documented PPCM case and 1 family member who died suddenly just after her second delivery, suggesting that this might also have been a case of PPCM.

PPCM Patients and Cardiological Screening of Their Family Members

We were able to evaluate data from 10 PPCM cases since the early 1990s. Treatment of PPCM patients was performed according to the guidelines for DCM patients. Five patients showed recovery of left ventricular function and dimensions beyond the criteria for DCM within 1 year, and we did not invite them to the cardiogenetics outpatient clinic. One patient was lost to follow-up, and another patient declined our invitation. The remaining 3 patients were seen at the cardiogenetics outpatient clinic. Cardiological screening of their first-degree family members revealed undiagnosed DCM in all 3 families (individuals 2a-II:2, 2b-II:2, and 2c-II:2). Detailed clinical data and the pedigrees of these 3 families are shown in Table 2 and Figure 2.

Genetic Analysis

We analyzed the DNA of the index patients of the 5 DCM families with at least 1 case of PPCM and of the 3 PPCM patients but found no mutations in *LMNA*, *TNNT2*, and *MYH7*. However, in family 1b, in which we performed more extensive DNA analysis, we identified a mutation in cardiac troponin C (c.149A>G, p.Gln50Arg in *TNNC1*; see Figure 3A). This mutation was seen in the index patient, in her affected mother (III:1), and in one of her 2 affected maternal great-aunts (II:4), implying that the grandmother (II:1) was an obligate carrier of this mutation. Individual II:5, an affected maternal great-aunt, refused DNA analysis. This mutation is believed to be pathogenic because (1) it alters a glutamine residue, which is highly conserved all the way up to the nematode *Caenorhabditis elegans* and is surrounded by conserved residues (see Figure 3B); (2) the amino acid substitution is localized in a small critical linker region (3 amino acids) between 2 calcium-binding/EF-hand (helix-loop-helix) domains known to be involved in protein-protein

Table 1. Clinical Features of Affected Members of DCM Families With PPCM Patients

Patient	Referred for	Diagnosis	Age at Diagnosis	Timing at Diagnosis	LVEF at Diagnosis, %	Pathology	Cardiological Remarks	Other Remarks	Follow-Up
1a-I:2	HF	DCM	51 y						
1a-II:1*	HF	PPCM	33 y	35th wk of pregnancy	23		Thrombus apex, sinus tachycardia	CS	LVEF 44% after 6 mo, stable for 4 y
1b-II:1	Died	DCM	54 y			Dilated heart, enlarged and hyperchromatic nuclei of the myocytes			
1b-II:2*	Died	SCD	26 y	Just after delivery		NA			
1b-II:4	Screening	Mild DCM	63 y						
1b-II:5	Screening	Mild DCM	62 y						
1b-III:1*	HF	PPCM	30 y	3 mo after delivery	21				No recovery after 9 mo
1b-IV:1	Heart murmur	DCM	16 mo						
1c-I:1	Died	DCM	63 y			NA			
1c-II:2	HF	DCM	41 y				LBBB		
1c-II:3*	HF	PPCM	26 y	Few days after delivery	NA	Dilated heart, myocyte hypertrophy, fibrosis	LBBB		Died after 2 wk of intractable HF
1c-III:1	Screening	Mild DCM	25 y						
1c-III:3	Screening	DCM	22 y						
1c-III:4	Screening	DCM	20 y						
1c-III:5	Screening	DCM	28 y						
1 d-II:1	Dyspnea	DCM	61 y						
1 d-II:3	Screening	DCM	61 y						
1 d-II:5*	HF	PPCM	29 y	Just after delivery	NA	NA			Died at 31 y of age
1 d-III:2*	Cardiogenic shock	PPCM	27 y	3 d after delivery	20	Mild hypertrophy of myocytes			Died within 1 mo of MOF
1 d-III:3	Screening	DCM	48 y						
1 d-III:4	Screening	DCM	48 y						
1e-II:1	AF	DCM	74 y				AF		
1e-II:3	Dyspnea	DCM	70 y				AF		
1e-III:2*	HF	PPCM	29 y	2 mo after delivery	23	EMB: signs of acute myocarditis, no autopsy	Developed AF	Suspicion of vasculitis, no primary APS	No recovery, died at 51 y of age of progressive HF

LVEF indicates left ventricular ejection fraction; HF, heart failure; CS, caesarean section; SCD, sudden cardiac death; LBBB, left bundle-branch block; MOF, multiorgan failure; AF, atrial fibrillation; EMB, endomyocardial biopsy; NA, not available; and APS, antiphospholipid syndrome.

*Presumed PPCM cases.

interactions (the p.Gln50Arg mutation probably perturbs normal function of this important domain); (3) the mutation cosegregates with disease in this family; (4) the mutation was absent in 300 alleles from ethnically matched control individuals; and (5) the mutation is classified as pathogenic by several prediction algorithms (Polyphen prediction: "probably damaging" [high confidence of affecting protein function or structure]; SIFT prediction: not tolerated [score, 0.00; SIFT scores range from 0 to 1, with 0 indicating the most deleterious mutation and 1 indicating the least deleterious mutation). Moreover, *TNNC1* is very highly conserved, and only a few sequence variants are found in this gene, which underpins our conclusion that the mutation in this family is pathogenic.

Discussion

This is the first study reporting a systematic approach to investigating the relation between PPCM and familial DCM. We identified a substantial number (5 of 90, 6%) of DCM families with PPCM patients. This number is considerably higher than would be expected by chance. Although the incidence of PPCM in the Netherlands is unknown, it has been reported that the incidence in the United States is only 1 in 4075 live births.[21] Second, in our exploratory study, undiagnosed DCM was identified in all 3 families of PPCM patients who did not show full recovery. Finally, the identification of a mutation (c.149A>G, p.Gln50Arg) in *TNNC1* in a DCM family with 1 PPCM patient and another family member who had died suddenly soon after a delivery underscores the genetic nature of this disease. Together, these findings strongly suggest that a subset of PPCM is part of the spectrum of familial DCM, presenting in the peripartum period. Hence, the statement from the European Society of Cardiology on the classification of cardiomyopathies, which classifies PPCM as a nonfamilial, nongenetic form of dilated cardiomyopathy associated with pregnancy,[7] may need to be reconsidered if more extensive multicenter studies confirm our findings.

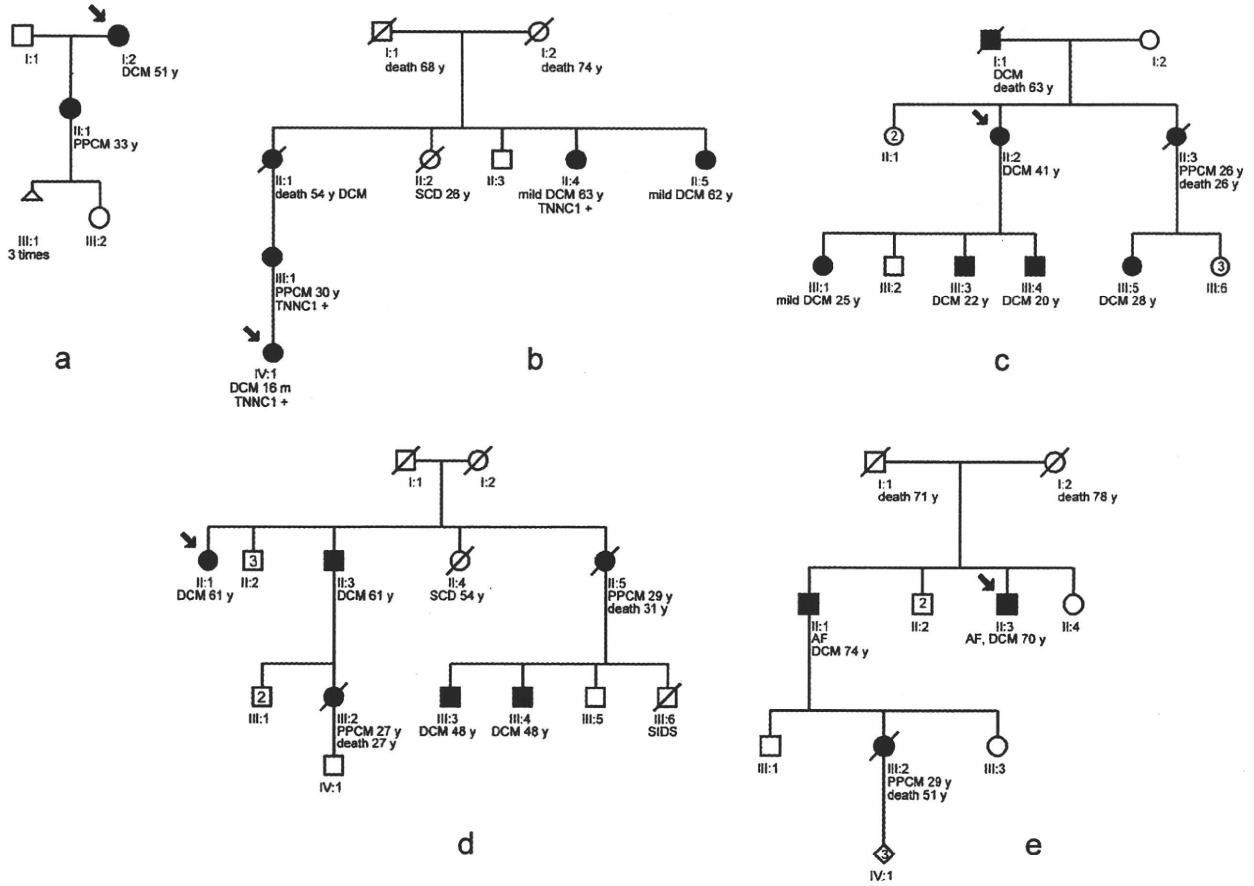

Figure 1. Pedigrees of DCM families with PPCM patients. Square symbols indicate men; circles, women; diamonds, unknown sex; and triangles, miscarriage. Solid symbols indicate clinical diagnosis of PPCM or (mild) DCM. Diagonal lines through symbols indicate deceased; arrow indicates index patient in each family; and the number in a symbol indicates the number of individuals with this symbol. AF indicates atrial fibrillation; SCD, sudden cardiac death; SIDS, sudden infant death syndrome; and TNNC1+, carrier of mutation p.Gln50Arg in *TNNC1* (cardiac troponin C).

Pathophysiology of PPCM and Role of Genetic Predisposition

Since its first description in 1849,[22,23] PPCM has remained an intriguing disease entity that often poses a significant challenge to the clinician. The diagnosis of PPCM is often delayed; an important reason for this delay is probably related to the fact that the cardinal symptoms of PPCM (fatigue, dyspnea, edema) are also associated with a normal pregnancy.[24] In addition, although the clinical course is often benign with recovery of left ventricular function and dimensions, a considerable subset of patients with PPCM progress to intractable heart failure necessitating heart transplantation.[25]

Table 2. Clinical Features of PPCM Patients and Their Affected Family Members Identified by Cardiological Screening

Patient	Referred for	Diagnosis	Age at Diagnosis, y	Timing at Diagnosis	LVEF at Diagnosis, %	Cardiological Remarks	Other Remarks	Follow-Up
2a-II:2	Screening, fatigue, palpitations	DCM	57					
2a-III:1*	HF	PPCM	33	37th wk of pregnancy	25		CS	LVEF 35% after 3 mo
2b-II:2	Screening	DCM	61					
2b-III:1*	HF	PPCM	23	Just after delivery	25		CS in 28th wk because of eclampsia	LVEF 10% after 8 y, CRT
2c-II:1*	TIA, PE	PPCM	33	2 mo after delivery	20	Thrombus in both ventricles	TIA, PE	LVEF 45% after 8 y
2c-II:2	Screening	Mild DCM	58					

LVEF indicates left ventricular ejection fraction; HF, heart failure; CS, caesarean section; CRT, cardiac resynchronization therapy; TIA, transient ischemic attack; and PE, pulmonary embolism.

*PPCM cases.

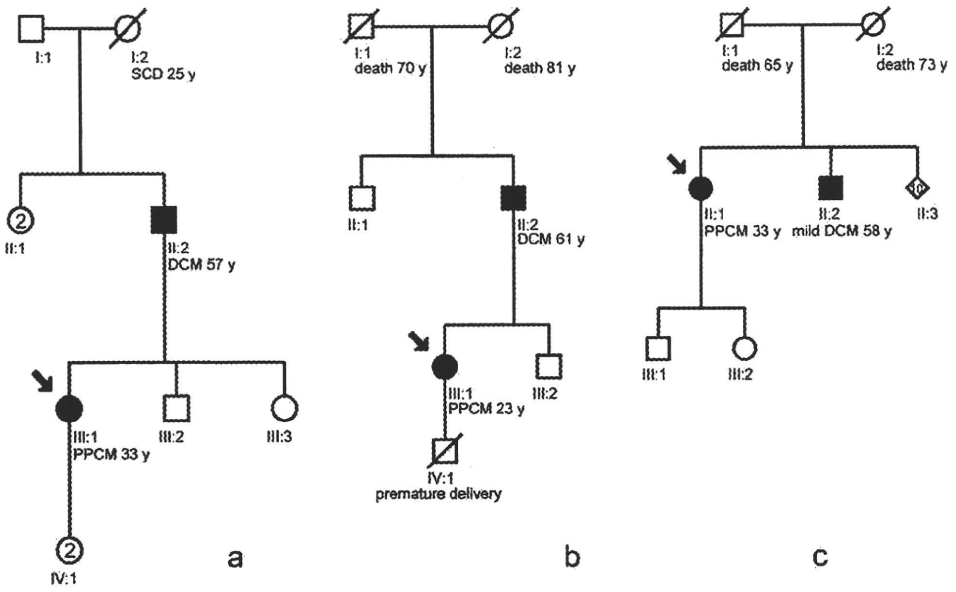

Figure 2. Pedigrees of the families of PPCM patients in which cardiological screening revealed undiagnosed cases of DCM. Square symbols indicate men; circles, women; and diamonds, unknown sex. Solid symbols indicate clinical diagnosis of PPCM or (mild) DCM. Diagonal lines through symbols indicate deceased; arrows indicate index patient in each family; and the number in a symbol indicates the number of individuals with this symbol. SCD indicates sudden cardiac death.

The cause of PPCM is still unresolved, but a unifying concept was recently proposed that explains several key features of PPCM. Oxidative stress rises during normal pregnancy, culminating in the last trimester; this runs parallel to an increase in antioxidant capacity with a peak early after delivery.[26] Several signaling pathways have been shown to be required for protection of the maternal heart, including c-Src-Akt and STAT3. In a mouse model, a deletion of *STAT3* caused proteolytic cleavage of prolactin into a potent antiangiogenic, proapoptotic, and proinflammatory factor associated with the development of PPCM.[7] The attractiveness of this concept notwithstanding, it does not explain why only a few women develop PPCM while the majority remain unaffected. As suggested by previous case reports, genetics may play a role: 4 reports describe a total of 8 kinships with >1 member with PPCM.[8–13] Moreover, 2 families have been reported with cases of PPCM and DCM in close relatives.[14,15]

In this study, we took a systematic approach to investigate the role of genetics in PPCM. We hypothesized that a subset of PPCM cases might occur against a background of familial DCM and found supporting evidence for this hypothesis. We identified PPCM cases in 5 of 90 families (6%) with DCM, and we found undiagnosed DCM in all 3 families of PPCM patients who did not show full recovery. It is also worth noting that 2 families (1b and 1d) probably had >1 member with PPCM.

In terms of pathophysiology, the following scenario has begun to emerge: There may be an interaction between environment and genetics in the sense that peripartum/postpartum oxidative stress ("environment") causes a genetically susceptible woman ("genetics") to cross the threshold toward overt DCM. More precisely, in women with a familial predisposition for DCM, the oxidative stress associated with the peripartum/postpartum period may trigger the manifestation of disease. However, it should be noted that familial predisposition can explain only part of the problem because even in DCM families only a minority of women develop PPCM.

Relative to the time window of PPCM, Elkayam and coworkers[27] reported that the clinical presentation and outcome of women with pregnancy-associated cardiomyopathy that was diagnosed early in pregnancy are comparable to those of women with formal PPCM. In fact, we also observed several cases of overt DCM before the last month of pregnancy in women from DCM families who had no prior

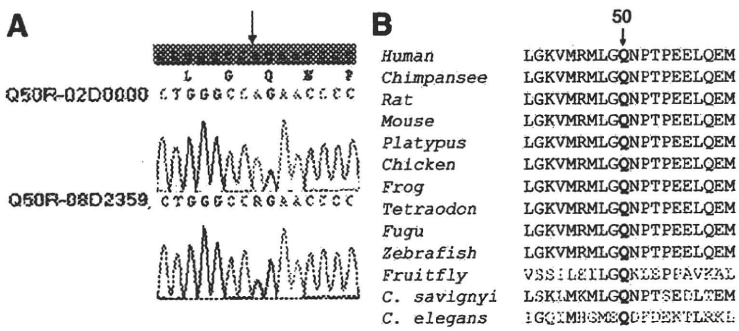

Figure 3. *TNNC1* mutation p.Gln50Arg. A, Sequence trace showing the presence of the p.Gln50Arg mutation in the index patient. Sequence trace of the relevant *TNNC1* fragment from a control DNA sample (02D0000) and from a sample of the index patient (08D2359) showing the A to G nucleotide change at position 149 (c.149A>G) leading to the missense mutation p.Gln50Arg. Mutation nomenclature according to www.HGVS.org. Arrow indicates nucleotide position 149. B, Sequence alignment showing cross-species conservation of the glutamine residue (Q) at position 50 of *TNNC1* up to the nematode *C elegans*.

history of heart disease (data not shown). In these instances, however, there is an alternative explanation, namely the hemodynamic challenge associated with pregnancy triggering overt DCM in the setting of subclinical DCM. To avoid any confusion, we adhered to the strict definition of PPCM, including the limited time window.

Genetic Analysis

We analyzed the genes believed to be most commonly involved in DCM (*LMNA*, *TNNT2*, and *MYH7*) but found no mutations. This is not surprising because the yield of DNA analysis in patients with DCM is, in general, still rather low (<20%), even when analyzing a relatively large batch of genes.[28] However, after extensive DNA analysis in family 1b, we did identify a mutation in *TNNC1* (p.Gln50Arg), supporting the genetic nature of the disease in this DCM family with a PPCM case. Moreover, this finding is important for the genetics of DCM in general because, to the best of our knowledge, only 1 germline *TNNC1* mutation (p.Gly159Asp) related to DCM has been reported in the literature so far.[29,30]

Disease expression in the family with the *TNNC1* mutation (p.Gln50Arg) is extremely variable (see Table 1). The index patient was diagnosed with DCM before 2 years of age; the mother developed PPCM after her first pregnancy; the maternal grandmother had no problems during her pregnancy, although she was diagnosed with DCM postmortem at 54 years of age; one of the grandmother's sisters died suddenly at 26 years of age just after her second delivery; and 2 other sisters of the grandmother were diagnosed with only mild DCM after screening but neither had problems during their pregnancies (3 and 2, respectively). In the previously reported DCM family with a *TNNC1* mutation (p.Gly159Asp), expression of the disease was severe, with several family members experiencing premature cardiac death or transplantation.[29] Remarkably, this family also had 1 member who had been diagnosed with DCM in childhood, whereas development of DCM in childhood is usually very rare in DCM families.[30] Further studies on the presence and effects of *TNNC1* mutations in PPCM patients and DCM families, including DCM diagnosed in childhood, are required to clarify the relationship between these conditions, the disease expression, and this gene.

Limitations

One important limitation of this study is that we did not perform cardiological screening of family members of those PPCM patients who had recovered left ventricular function and dimensions beyond the criteria for DCM. At the time we designed this study, we believed that we could not clinically or ethically justify offering presymptomatic cardiological screening to these families because the chances of identifying clinically relevant findings seemed smaller than the possible side effects (like psychological stress, unforeseen diagnostic findings outside this context, and problems with health insurance) that might arise from such screening. However, given our results, it would be interesting to study these families because these PPCM cases might also be part of familial DCM.

Another limitation is the small number of PPCM cases in this study. The finding of undiagnosed DCM in all 3 analyzed families of PPCM patients may be overrepresented, and larger numbers are necessary to assess the real incidence of this phenomenon. Therefore, a more extensive multicenter, systematic cardiological screening study of first-degree relatives of PPCM patients, including the relatives of recovered PPCM patients, is important. This would provide the opportunity to confirm our results and to study the relation of PPCM and familial DCM in more detail.

It is notable that the outcome of the 6 PPCM cases in the DCM families appears to be worse (see Table 1) than the known characteristics of PPCM in general, in which a subset has a benign course with recovery of left ventricular function and dimensions. Larger numbers of cases are needed to allow a comparison of the clinical characteristics of PPCM cases in DCM families with isolated PPCM cases.

Conclusions and Practical Implications

To the best of our knowledge, this is the first systematic study of the relation between PPCM and familial DCM. We found cases of PPCM in 5 of 90 DCM families and identified previously undiagnosed DCM in the families of all 3 PPCM patients who did not show a full recovery. We found support for the genetic nature of disease in 1 DCM family with PPCM by identifying a mutation in *TNNC1*. We therefore conclude that PPCM can be a manifestation of familial DCM.

Obviously, further research is needed to confirm our findings, for instance, to better understand the interaction between oxidative stress associated with the peripartum/postpartum period and genetics. However, our findings already have several practical implications. First, because PPCM can be the first manifestation of familial DCM, we recommend presymptomatic screening for covert DCM in first-degree family members of PPCM patients without recovery of left ventricular function and dimensions. Second, as part of routine procedures, we already follow healthy women (ie, without proven previous signs of DCM) during pregnancy if they are first-degree family members of affected individuals in a DCM family, but this monitoring should be extended to the puerperium.

Acknowledgments

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Disclosures

None.

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CLINICAL PERSPECTIVE

Anecdotal cases with familial clustering of peripartum cardiomyopathy (PPCM) or joint occurrence of PPCM and idiopathic dilated cardiomyopathy (DCM) within families have been reported. We performed the first systematic study of the relation between PPCM and familial DCM. In a substantial number of DCM families (5/90), cases of PPCM were found, and more important, previously undiagnosed cases of DCM were identified in all 3 families of PPCM patients who did not show a full recovery. Moreover, our results support the genetic nature of the disease in 1 DCM family with PPCM: We identified a mutation in the gene encoding cardiac troponin C (*TNNC1*). We therefore conclude that PPCM can be a manifestation of familial DCM. Obviously, further research is needed to confirm these observations in a larger series of patients. However, our findings already have several clinical implications and may change clinical practice and thinking about PPCM. Because PPCM can be the first manifestation of familial DCM, we recommend presymptomatic cardiological screening for covert DCM in first-degree family members of PPCM patients without recovery of left ventricular function and dimensions. Second, cardiological screening during pregnancy should be considered for healthy women (ie, without proven previous signs of DCM) who are first-degree family members of familial DCM patients and this should be extended into the puerperium.

Cell

A Cathepsin D-Cleaved 16 kDa Form of Prolactin Mediates Postpartum Cardiomyopathy

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SUMMARY

Postpartum cardiomyopathy (PPCM) is a disease of unknown etiology and exposes women to high risk of mortality after delivery. Here, we show that female mice with a cardiomyocyte-specific deletion of *stat3* develop PPCM. In these mice, cardiac cathepsin D (CD) expression and activity is enhanced and associated with the generation of a cleaved antiangiogenic and proapoptotic 16 kDa form of the nursing hormone prolactin. Treatment with bromocriptine, an inhibitior of prolactin secretion, prevents the development of PPCM, whereas forced myocardial generation of 16 kDa prolactin impairs the cardiac capillary network and function, thereby recapitulating the cardiac phenotype of PPCM. Myocardial STAT3 protein levels are reduced and serum levels of activated CD and 16 kDa prolactin are elevated in PPCM patients. Thus, a biologically active derivative of the pregnancy hormone prolactin mediates PPCM, implying that inhibition of prolactin release may represent a novel therapeutic strategy for PPCM.

INTRODUCTION

Postpartum cardiomyopathy (PPCM) is a disease of unknown etiology, characterized by an acute onset of heart failure in women in the late stage of pregnancy up to several months postpartum, resulting in high mortality despite optimal medical therapy (Elkayam et al., 2005; Reimold and Rutherford, 2001; Sliwa et al., 2002).

In PPCM patients, serum markers of inflammation and apoptosis are significantly elevated, which appears to predict impaired functional status and mortality, consistent with the idea that inflammation and apoptosis may contribute to the pathogenesis of PPCM (Sliwa et al., 2006; Sliwa et al., 2002). In line with these clinical observations, it has been shown that transgenic mice with cardiac-specific overexpression of the α subunit of Gq develop PPCM, which could be attenuated by pharmacological inhibition of apoptosis (Hayakawa et al., 2003).

We noted that female mice with a homozygous or heterozygous cardiomyocyte-specific knockout of STAT3 (*αMHC-cre*$^{+/o}$*; stat3*$^{flox/flox}$: CKO; *αMHC-cre*$^{+/o}$*; stat3*$^{flox/+}$: HET) develop PPCM in a dose-dependent manner (CKO > HET). Notably, STAT3 is involved in protection of the heart from oxidative stress by upregulation of antioxidative enzymes such as the reactive oxygen species (ROS) scavenging enzyme manganese superoxide dismutase (MnSOD) (Negoro et al., 2001). STAT3 also plays an important role in promoting myocardial angiogenesis both by paracrine and autocrine mechanisms in cardiomyocytes and nonmyocytes (Bartoli et al., 2003; Hilfiker-Kleiner et al., 2004a; Osugi et al., 2002), and it can mediate cardiomyocyte hypertrophy (Hilfiker-Kleiner et al., 2004a; Kunisada et al., 2000). In the present study, we used the above genetic mouse model to investigate potential underlying mechanisms, which may initiate and/or drive PPCM. We found a detrimental link between enhanced oxidative stress and cleavage of the pregnancy hormone prolactin (PRL) into an antiangiogenic 16 kDa form (Corbacho et al., 2002; Tabruyn et al., 2003) as a major cause of

Table 1. HW:BW, Cardiac Dimensions, and Function in NT and CKO Female Mice Postpartum with or without Treatment with BR

	NP		PP		PP/BR	
	NT	CKO	NT	CKO	NT	CKO
HW:BW (mg:g)	3.8 ± 0.5	3.7 ± 0.3	4.6 ± 0.5*	5.5 ± 1.2*#	4.1 ± 0.5	4.5 ± 0.7**
LVEDD (mm)	3.5 ± 0.2	3.6 ± 0.4	3.9 ± 0.2**	4.8 ± 1.0**#	3.9 ± 0.2*	3.6 ± 0.2
LVESD (mm)	2.2 ± 0.3	2.4 ± 0.5	2.7 ± 0.4**	3.8 ± 1.3**#	2.5 ± 0.1*	2.3 ± 0.3
IVSD (mm)	0.69 ± 0.07	0.69 ± 0.10	0.72 ± 0.19	0.80 ± 0.21	0.65 ± 0.06	0.62 ± 0.08
IVSS (mm)	1.1 ± 0.1	1.1 ± 0.1	1.2 ± 0.2	0.9 ± 0.4#	1.1 ± 0.1	1.1 ± 0.2
LVPWD (mm)	0.53 ± 0.11	0.50 ± 0.08	0.64 ± 0.11*	0.50 ± 0.05	0.50 ± 0.08	0.52 ± 0.08
LVPWS (mm)	0.97 ± 0.10	0.87 ± 0.12	0.98 ± 0.25	0.58 ± 0.20*#	0.90 ± 0.14	0.80 ± 0.16
FS (%)	38 ± 3	33 ± 5	32 ± 7*	14 ± 8**##	38 ± 4	36 ± 9
HR (beats/min)	350 ± 54	343 ± 74	312 ± 31	326 ± 72	300 ± 31	303 ± 54

Postpartum (PP), postpartum/BR (PP/BR), and age-matched nulli-pari (NP) females were utilized for these studies. Heart-to-body-weight ratio (HW:BW), LV end-diastolic diameter (LVEDD), end-systolic diameter (LVESD), intraventricular septum thickness in diastole (IVSD) or in systole (IVSS), LV posterior wall thickness in diastole (LVPWD) and systole (LVPWS), fractional shortening (FS), and heart rate (HR) were determined by transthoracic echocardiography in sedated mice. At least five animals per group were investigated for each parameter. Data are represented as mean ± SD, *$p < 0.05$, **$p < 0.01$ versus NT-NP, #$p < 0.05$, and ##$p < 0.01$ versus NT-PP.

PPCM. Furthermore, we explored similarities between PPCM in mice and humans and initiated a novel therapeutic strategy with the PRL inhibitor bromocriptine in woman with a high risk of developing PPCM.

RESULTS

Cardiac-Restricted Deletion of *stat3* Leads to PPCM

Nulli-pari CKO (CKO-NP) and HET females (HET-NP) develop normally and do not show signs of heart failure or increased mortality. At the age of 6 months, cardiac function is normal and comparable with sisters harboring two copies of the floxed *stat3* allele (NT-NP) without the *αMHC-cre*$^{+/o}$ transgene (Table 1, HET-NP, data not shown).

No pregnancy-associated cardiac phenotype or mortality was observed in 20 NT sisters (Figure 1A) or ten females with only the *cre* transgene (*αMHC-cre*$^{+/o}$: C+/+-PP; Figure 1A and Table S1, in the Supplemental Data available with this article online). Thus, neither the floxed *stat3* allele alone nor *cre* alone had an adverse effect peri- and postpartum. By contrast, physiological stresses of pregnancy, labor, and/or nursing resulted in PPCM in all of the 53 CKO females studied. Roughly two-thirds (37 out of 53 CKO females versus 0 out of 20 NT-PP females, $p < 0.01$) had died after the second pregnancy (Figure 1A). Notably, no CKO female survived more than five pregnancies. In HET females, PPCM-related death was found after three to four deliveries (Figure 1A). Death occurred always within the first 3 weeks after delivery. After two and four pregnancies, respectively, the majority of CKO and HET females presented signs of overt heart failure, such as generalized edema and labored breathing. The hearts were characterized by four-chamber dilatation, often with thrombi in the atria, extensive fibrosis (Figure 1B), and an increase in cardiomyocyte length (Table S2 and Figure S1). Echocardiography revealed left ventricular (LV) dilatation and depressed fractional shortening in CKO-PP and HET-PP females (Table 1 and Table S3) compared with NT-PP females. CKO-PP females showed increased cardiac mRNA levels of hypoxia inducible factor-1α (HIF1α) (Chi and Karliner, 2004) and BNIP3 (Kubasiak et al., 2002) (Figure 1C) and a markedly reduced content of energy-rich phosphates, ATP (−68% ± 7%, $p < 0.01$ versus NT-PP), and ADP (−57% ± 10%, $p < 0.01$ versus NT-PP), indicative for cardiac hypoxia. Cardiac apoptosis, as indicated by an increased number of TUNEL-positive cells (cardiomyocytes and nonmyocytes) and protein levels of activated caspase-3 (act-Casp-3), was substantially higher in CKO-PP females compared with NT-PP females (Figures 1D and 1E) or C+/+-PP females (Figure S2).

STAT3 Is Activated in the Maternal Heart in Pregnancy and Postpartum

In NT-Prg (day 17 of pregnancy) and NT-PP females, we observed increased Tyr-705 phosphorylation of STAT3 (Figure 1F). As expected, cardiac STAT3 was barely detectable in CKO females (Figure 1F) and STAT3 signals were markedly reduced in the isolated cardiomyocyte-enriched fraction, but not in the nonmyocyte fraction of CKO-PP compared with NT-PP hearts, (Figure S2), indicating that STAT3 activation in LVs from NT-PP mice is mainly occurring in cardiomyocytes. Prolactin (PRL) serum levels increased late in pregnancy and postpartum and are known to activate STAT3 (Cataldo et al., 2000). In fact, infusion of recombinant PRL activated STAT3 in the heart in vivo, and addition of PRL to cultured cardiomyocytes activated STAT3 in vitro (Figure S3).

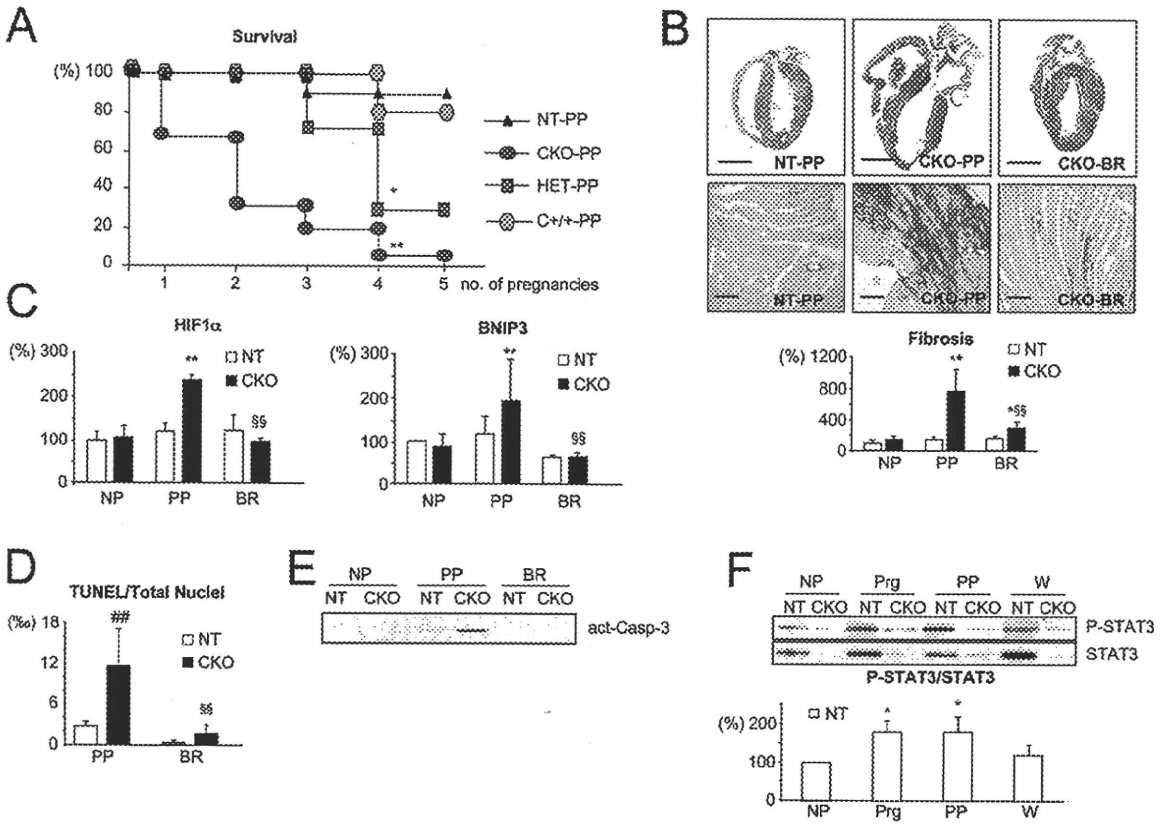

Figure 1. PPCM in CKO-PP Mice

(A) Survival in relation to number of pregnancies of NT-PP (n = 20), C+/+-PP (n = 10), HET-PP (n = 7), and CKO-PP mice (n = 53).

(B) Representative H&E (upper panels) and picro-Sirius red (lower panels) staining in heart sections from a CKO-PP mouse (middle panels), an age- and pregnancy-matched NT-PP mouse (left panels), or a CKO-BR mouse (right panel). Bars in upper panels, 2.5 mm; bars in lower panels, 100 μm. Bar graph summarizes quantification of fibrosis.

(C and D) Bar graphs summarizing mRNA expression of HIF1α and BNIP3 (C) or the ratio of TUNEL positive to total number of nuclei in LVs from NT and CKO females (D).

(E) Protein levels of act-Casp-3 in LVs from NT or CKO females.

(F) Protein levels of P-STAT3 and total STAT3 in LVs from NT or CKO mice; the bar graph summarizes the ratio of P-STAT3:STAT3 in LVs from NT females.

In (B)–(H), four to seven mice per group were analyzed. Abbreviations: NP, nulli-pari; Prg, pregnant; PP, postpartum; and W, 4 weeks after weaning. Data are presented as mean ± SD. *p < 0.05, **p < 0.01 versus NT-NP, ##p < 0.01 CKO-PP versus NT-PP, and §§p<0.01 CKO-BR versus CKO-PP.

Cardiac Deficiency of STAT3 Does Not Affect Pregnancy-Induced Cardiac Hypertrophy

Pregnancy induces a reversible physiological hypertrophy of cardiomyocytes (Eghbali et al., 2005). CKO-Prg and NT-Prg females showed a similar increase in cardiomyocyte growth (cross-sectional area and length; Figure S1 and Table S2), indicative for normal Prg-induced cardiac hypertrophy in CKO-Prg females.

Postpartum Myocardial Angiogenesis Is Impaired in CKO Females

Physiological hypertrophy, as it is observed during pregnancy (Eghbali et al., 2005), requires the proportional growth of the capillary network (Hudlicka and Brown, 1996). In NT-Prg and CKO-Prg females, the ratio of capillaries to cardiomyocytes increased to a similar degree (Figures 2A and 2B). This increased capillary density was maintained in NT-PP females, although it had decreased to prepregnancy levels in CKO-PP females (Figures 2A and 2B). The reduction in the LV capillary density in CKO-PP mice was paralleled with decreased expression of VEGF (CKO-PP: mRNA, −30% ± 15%; VEGF protein: −56% ± 22% versus NT-PP, $p < 0.05$) and of von Willebrandt Factor (CKO-PP: vWF mRNA, −26% ± 16% versus NT-PP, $p < 0.05$).

Increased Oxidative Stress in CKO-PP Hearts

STAT3 is known to protect cardiomyocytes from oxidative stress in part by the upregulation of the ROS scavenging enzyme MnSOD (Negoro et al., 2001). NT-PP mice showed a marked increase in cardiac MnSOD protein levels compared to NT-NP, whereas only a moderate increase was

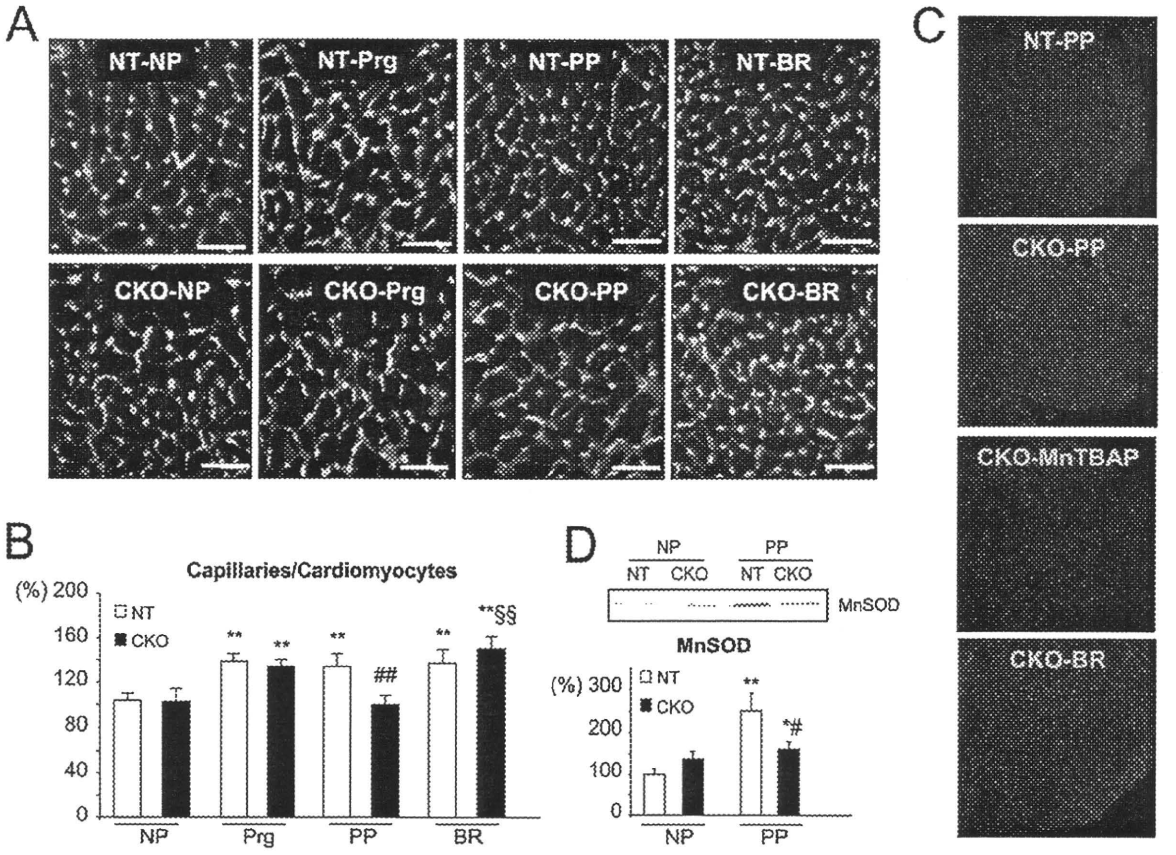

Figure 2. Capillary Density in the Peri- and Postpartum Heart

(A) Capillaries in LV sections of NT-NP, CKO-NP, NT-Prg, CKO-Prg, NT-PP, CKO-PP, NT-BR, and CKO-BR were identified by isolectin B4 immunohistochemistry (yellow). WGA marks cell membranes (red), and Hoechst stain identifies nuclei (blue) (bars, 40 μm).

(B) Bar graph summarizing capillaries per 100 cardiomyocytes in NT and CKO mice; ratios in NT-NP females were set as 100% (CKO-NP, n = 12; NT-Prg, n = 4; CKO-Prg, n = 5; NT-PP, n = 12; CKO-PP, n = 12; NT-BR n = 11; and CKO-BR, n = 11). Similar data were obtained with CD31/WGA staining (data not shown).

(C) In situ detection of superoxide production with dihydroethidium fluorescence staining (the oxidative dye hydroethidine is red fluorescent when oxidized to EtBr by O_2^-) in LV sections of NT-PP, CKO-PP, CKO-MnTBAP, and CKO-BR females 5 days postpartum.

(D) Protein levels of MnSOD in NT and CKO mice; the bar graph summarizes MnSOD protein levels (n = 4–6 each genotype). Data are presented as mean ± SD, *p < 0.05, **p < 0.01 versus NT-NP, #p < 0.05, ##p < 0.01 CKO-PP versus NT-PP, and §§p < 0.01 CKO-BR versus CKO-PP.

observed in CKO-PP females (Figure 2D). Likewise, cardiac MnSOD mRNA levels were higher in NT-PP than CKO-PP females (NT-PP: +54% ± 1% versus CKO-PP, p < 0.05). The production of ROS was enhanced in LVs from CKO-PP compared with NT-PP females as determined by dihydroethidium fluorescence staining, (5 days postpartum: +91% ± 32% versus NT-PP, p < 0.05, Figure 2C; and 3 weeks postpartum CKO-PP: +48% ± 23% versus NT-PP, p < 0.05) and as determined by NADH-stimulated superoxide production measured by electron spin resonance spectroscopy (3 weeks postpartum: CKO-PP: +122% ± 71% versus NT-PP, p < 0.05).

MnTBAP Attenuates PPCM in CKO Mice

We assessed whether the pharmacological suppressor of ROS, tetrakis (4-benzoic acid) porphyrin (MnTBAP), a substance with catalytic activities similar to MnSOD (Houstis et al., 2006), would prevent PPCM. In fact, MnTBAP attenuated ROS generation in CKO females (CKO-MnTBAP: +6% ± 7% versus NT-PP, n.s.; Figure 2B), the upregulation of activated cathepsin D (CD) and act-Casp-3 protein, and the increase in β-myosin heavy chain (βMHC) mRNA (Figure S4). Moreover, CKO-MnTBAP females showed preserved cardiac capillary density (Figure S4) and cardiac function (Table S4) and displayed no postpartum-related mortality (0% in CKO-MnTBAP, n = 12, after two subsequent pregnancies). Despite preserved cardiac function, MnTBAP did not prevent the LV dilatation (Table S4) and the upregulation of matrix metalloproteinase 3 (MMP3) mRNA (Figure S4), indicating only a partial suppression of the PPCM phenotype in CKO females.

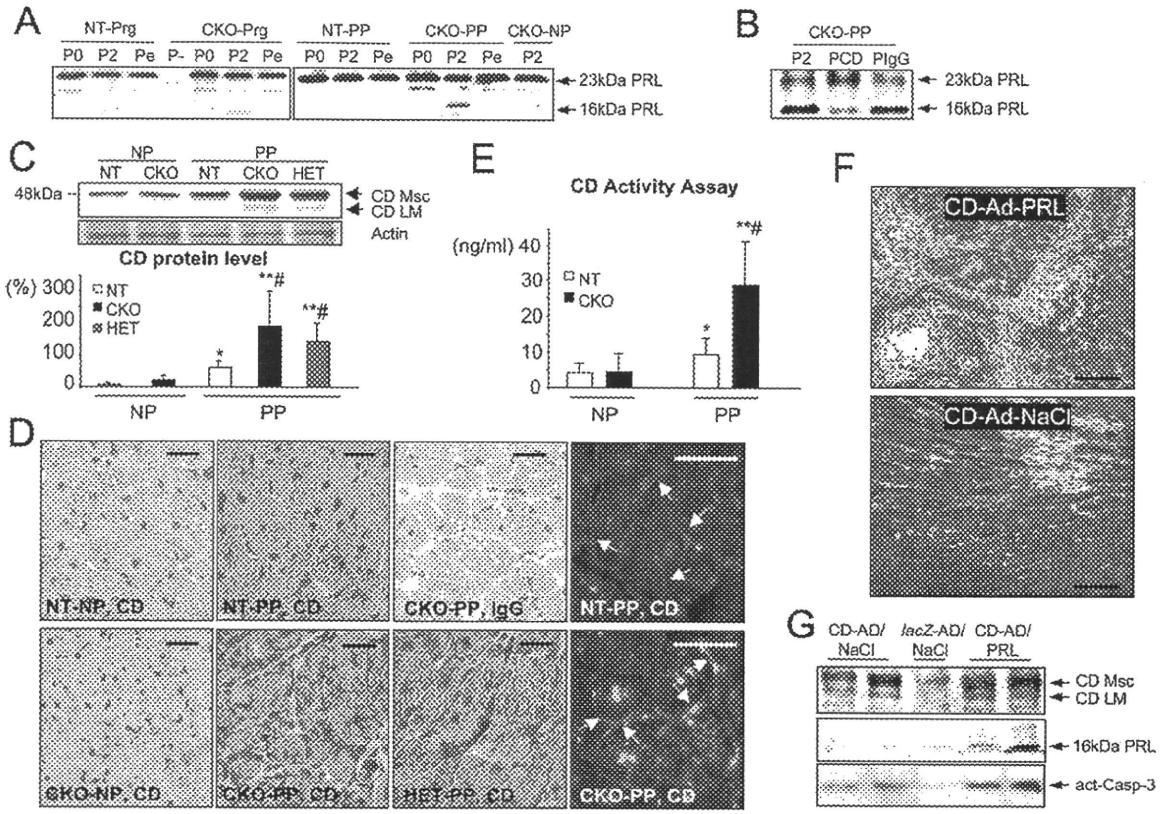

Figure 3. PRL Cleavage Assay and CD Expression and Activity in LVs of NT and CKO Females

(A) Protein levels of 23 and 16 kDa PRL in NT-Prg, CKO-Prg, NT-PP, CKO-PP, or CKO-NP LV supernatant after incubation with recombinant PRL for 0 hr (P0) or for 2 hr (P2) in the absence or presence of pepstatin A (Pe) or (B) after preabsorbtion with a neutralizing CD antibody (PCD) or unspecific IgG antibody (PIgG) prior to incubation for 2 hr.

(C) LV protein levels of CD (CD Msc, mature single-chain; CD LM, large chain of mature double-chain); the bar graph summarizes CD protein levels, and (D) anti-CD staining (brown) and nuclear staining with hematoxylin (blue) in LV sections form in NT, CKO and HET females, NP, and PP. IgG served as control for anti-CD specificity. Right panels demonstrate extracellular localization (arrows point to interstitium) of CD in CKO-PP by immunofluorescence staining in a larger magnification (CD, green; WGA, red; and Hoechst, blue); bars, 50 μm.

(E) Bar graph summarizing CD activity in NT and CKO LV supernatant.

(F) H&E-stained LV section from NT-NP mice infected with CD-Ad in the presence (CD-Ad-PRL) or absence (CD-Ad-NaCl) of high levels of recombinant PRL.

(G) Protein levels of CD, 16 kDa PRL, and act-Casp-3 in LVs from CD-Ad-NaCl, *lacZ*-Ad-NaCl, and CD-Ad-PRL mice. NP, nulli-pari; PP, postpartum. All data are from n = 4–6 individuals per genotype and are presented as mean ± SD. *$p < 0.05$, **$p < 0.01$ versus NT-NP, and #$p < 0.05$ CKO-PP or HET-PP versus NT-PP. HET-PP mice after four litters.

CD-Dependent Cleavage of PRL in CKO-PP Hearts

PRL, a dominant hormone in pregnancy and early postpartum, can exert opposing effects on angiogenesis depending on proteolytic processing of the proangiogenic full-length 23 kDa PRL into an antiangiogenic 16 kDa form (Corbacho et al., 2002; Tabruyn et al., 2003). An antibody recognizing both forms of PRL (Lkhider et al., 2004) showed immunoreactivity in LV sections of nursing CKO-PP and NT-PP females, but not in LV sections of NT-NP or CKO-NP females (Figure S5), whereas in western blot analysis, neither this nor other commercially available antibodies against PRL revealed reproducible quantitative or qualitative results. However, in an ex vivo assay (Lkhider et al., 2004) using supernatant of minced LV tissue from CKO-PP females, a high cleavage activity for recombinant PRL into the 16 kDa form was observed, whereas no cleaved 16 kDa PRL was generated from LV tissue extracts of CKO-NP, NT-PP, CKO-Prg, or NT-Prg mice (Figure 3A).

Among the proteolytic enzymes known to process PRL, CD very efficiently cleaves PRL into its 16 kDa form (Corbacho et al., 2002; Tabruyn et al., 2003). In fact, the PRL cleaving activity of CKO-PP LV supernatant was attenuated by preincubation with the CD inhibitor pepstatin A (Lkhider et al., 2004) or by preabsorbing the supernatant with a CD neutralizing antibody (Figures 3A and 3B), implicating that CD is the major PRL cleavage enzyme in CKO-PP hearts.

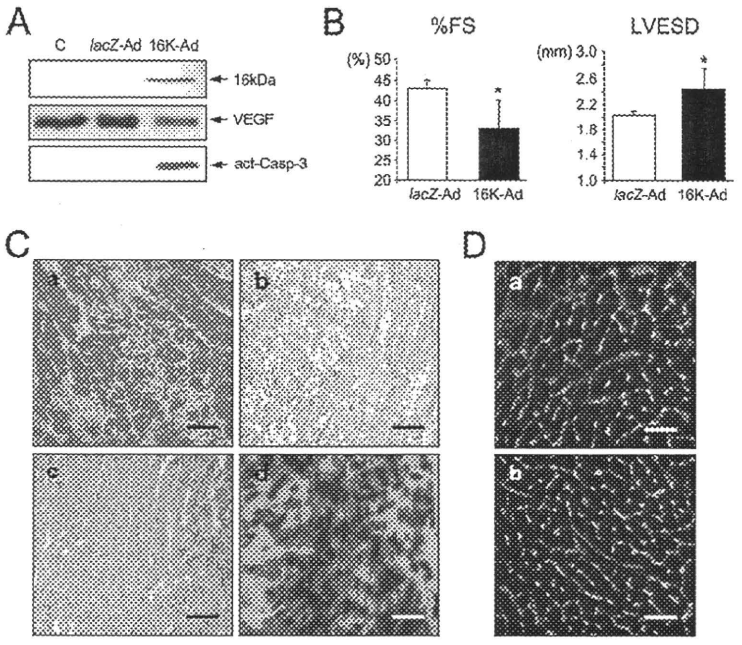

Figure 4. Adenoviral Expression of 16 kDa PRL in Mouse LVs

(A) Protein expression of 16 kDa PRL, VEGF, and act-Casp-3 in LVs from NT females or controls (C) infected with 16K-Ad or *lacZ*-Ad.

(B) Bar graphs display reduced percentage of FS and LV dilatation in NT-NP females infected with 16K-Ad (n = 5) compared with females infected with *lacZ*-Ad (n = 5).

(C) Anti-PRL staining (brown, [a]) and IgG control (b) in 16K-Ad-infected LV sections. Anti-PRL (c) or *lacZ* (d) staining in *lacZ*-Ad-infected LV tissue sections (bars, 100 μm).

(D) Capillary density identified by staining with isolectin B4 (yellow), WGA (red), and Hoechst (blue) in LV sections from 16K-Ad (a) or *lacZ*-Ad NT-NP females (b) (bars, 50 μm). Data are presented as mean ± SD, *p < 0.05.

Oxidative stress promotes the release of CD from lysosomes into the cytosol in cardiomyocytes (Corbacho et al., 2002; Roberg and Ollinger, 1998). Cardiac protein levels of the active single chain mature form of CD (CD Msc) were markedly upregulated in CKO-PP females compared with those in NT-PP, NT-NP, and CKO-NP females (Figure 3C), but expression of activated CD was attenuated in CKO-MnTBAP females (Figure S4). LV sections from NT-NP and CKO-NP females showed weak staining for CD in cardiomyocytes (Figure 3D), whereas marked staining for CD was observed in cardiomyocytes and the LV interstitium of CKO-PP and HET-PP females (Figure 3D). CD cleavage activity was substantially enhanced in supernatants of minced LVs of CKO-PP females compared with CKO-NP or NT-PP females (Figure 3E). Cultured cardiomyocytes infected with a CD expressing adenovirus (CD-Ad) released active CD forms into the cell culture medium, which processed 23 kDa PRL into 16 kDa PRL (Figure S6).

Prolactin Promotes Cardiac Injury in CD Overexpressing Hearts

We analyzed the effect of locally produced CD in the presence of high systemic PRL levels by injecting a CD expressing adenovirus (CD-Ad) into hearts of NT-PRL or control females (NT-NaCl). Seven days after infection with CD-Ad, NT-PRL mice showed severe cardiac damage (Figure 3F), including elevated levels of act-Casp-3 (Figure 3G) and enhanced mortality compared with NT-NaCl mice infected with CD-Ad (mortality: CD-Ad in NT-PRL, 33%, n = 9; versus 0% in CD-Ad in NT-NaCl, n = 7). Enhanced cardiac CD expression was detected in all CD-Ad-infected mice. PRL, notably mainly the cleaved 16 kDa form, could only be detected in females that were chronically infused with recombinant 23 kDa PRL (Figure 3G).

16 kDa PRL Decreases Myocardial Capillary Density and Reduces Cardiac Function Independent of Pregnancy

To examine the effect of the 16 kDa PRL in the heart, we injected adenoviral vectors expressing human 16 kDa PRL (16K-Ad) or *lacZ* (*lacZ*-Ad) as control into the LV wall of NT-NP females. Two weeks after infection, persistent cardiac expression of 16 kDa PRL was observed in 16K-Ad-infected females that was associated with LV dilatation and decreased cardiac function compared with *lacZ*-Ad-infected females (Figures 4A–4C). The 16 kDa PRL expression was paralleled by decreased cardiac capillary density (−31% ± 12%, $p < 0.05$, Figure 4D), reduced VEGF expression, and increased protein levels of act-Casp-3 (Figure 4A). Infection with 16K-Ad did not alter the LV:BW ratio or the cardiomyocyte CSA (Figure S6). In cultured cardiomyocytes, survival was similar after infection with the 16K-Ad or the *lacZ*-Ad (Figure S6). However, 16K-Ad infection of cultured cardiomyocytes impaired their metabolic activity (Figure S6).

Chronic infusion (3 weeks) of recombinant full-length 23 kDa PRL in NT-NP and CKO-NP (NT-PRL and CKO-PRL) in absence of postpartum-associated stress did not affect cardiac function and survival (Table S5). Activation of STAT5 was observed in LVs of NT-PRL and CKO-PRL mice (Figure S3), in line with studies implicating STAT5 as a preferential target for PRL (Cataldo et al., 2000). In addition, stimulation with 23 kDa PRL enhanced the activation of STAT3 in NT-NP hearts in vivo and in cultured cardiomyocytes in vitro (Figure S3). In line with described

proangiogenic effects of 23 kDa PRL (Corbacho et al., 2002), the myocardial capillary density was slightly enhanced in NT-PRL and CKO-PRL females (Table S5).

Bromocriptine, an Inhibitor of PRL Secretion, Prevents PPCM in CKO Females

The role of PRL for the development of PPCM was tested by preventing its release from the pituitary glands using bromocriptine (BR), a dopamine-D2-receptor agonist, known to block PRL efficiently in humans (Harrison, 1979) and mice (Nagafuchi et al., 1999). CKO (CKO-BR) and NT (NT-BR) females were treated with BR for two consecutive pregnancies. The efficient blockade of PRL release was confirmed by the following observations: (1) in contrast to NT-PP and CKO-PP females with detectable PRL antigens in LV sections, LV sections from NT-BR and CKO-BR mice were negative for PRL staining (Figure S5), (2) NT-BR and CKO-BR were not able to nurse their offspring because pups appeared underdeveloped three days after birth, and (c) the increased activation of STAT5, the preferential target of PRL, was attenuated in CKO-BR and NT-BR females (Figure S7).

BR prevented postpartum mortality in CKO-BR females (mortality after two pregnancies: 0% in CKO-BR females, n = 12 versus 70% in CKO-PP females, n = 53, $p < 0.01$) and preserved postpartum angiogenesis (Figures 2A and 2B), cardiac function, and dimensions (Figure 1B, Table 1, and Figure S8). BR also prevented cardiac fibrosis and apoptosis (Figures 1B, 1D, and 1E), attenuated the mRNA expression of HIF1α and BNIP3 (Figure 1C) and βMHC and MMP3 (Figure S8), and normalized the expression of αMHC (Figure S8) in CKO-BR females.

BR did not reduce ROS production in the early state (5 days postpartum) of treatment (CKO-BR: +67% ± 9% versus CKO-PP: 91% ± 32%, n.s., Figure 2C). After 3 weeks of postpartum treatment, however, ROS production in CKO-BR was markedly reduced (CKO-BR: +7% ± 23% versus CKO-PP 48% ± 23%, $p < 0.05$, Figure S8). In NT-BR females, cardiac function, cardiac angiogenesis, blood pressure, and heart frequency were not affected (Table 1 and Tables S6–S8). Thus, we did not identify direct effects of BR on the myocardium.

Decreased STAT3 Protein Levels in the Myocardium of PPCM Patients

To explore whether STAT3 is involved in human PPCM as well, STAT3 protein levels were quantified in LV tissue obtained from PPCM patients (n = 5) at the time of transplantation and compared with levels in similar LV tissues of otherwise normal human hearts (n = 7). Figure 5A shows reduced STAT3 protein levels in LVs from patients with PPCM compared with normal human LVs.

Serum of Lactating Women with PPCM Displays Increased oxLDL Levels, Enhanced Activation of CD, and Augmented Protein Levels of the 16 kDa PRL

In the serum of lactating patients with PPCM, levels of oxidized low-density lipoprotein (oxLDL), a marker for oxidative stress (Weinbrenner et al., 2003), and CD activity were enhanced compared with healthy lactating mothers (Figures 5B and 5C). Marked levels of an antigen corresponding to the 16 kDa PRL were detected by western blot (Figure 5D) in the sera of three out of five lactating PPCM patients with obvious cardiac dysfunction at the time of serum sampling (mean percentage of EF, 24 ± 7). It is important to note that the 23 kDa form of PRL was readily detectable, whereas the 16 kDa PRL was barely detectable in healthy lactating women (n = 5, Figure 5D).

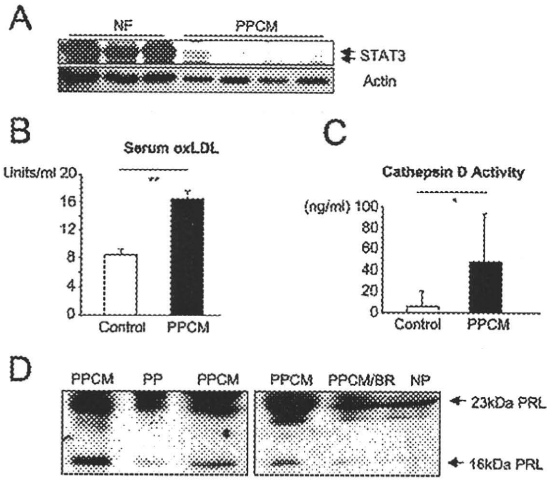

Figure 5. Cardiac STAT3 Expression and Serum Levels of Activated CD and Cleaved 16 kDa PRL in Patients with PPCM

(A) STAT3 protein levels in nonfailing (NF) LVs and in LVs from patients with end-stage heart failure due to PPCM. Total actin served as loading control.

(B) Serum levels of oxLDL in PPCM patients (n = 29) and pregnancy matched controls (n = 21).

(C) CD activity in nursing PPCM patients (n = 5) and in pregnancy matched healthy control women (n = 5).

(D) PRL immunoprecipitation followed by detection of 23 kDa and 16 kDa PRL in serum of nursing PPCM patients, healthy nursing women (PP), control women (NP), and women with PPCM after a 14 days treatment with BR (PPCM/BR). Data are presented as mean ± SD, $^{*}p < 0.05$, and $^{**}p < 0.01$.

Indication that Bromocriptine Treatment Prevents PPCM in High-Risk Patients

Women with PPCM who recover normal cardiac function have a high risk for recurring PPCM (Sliwa et al., 2004). We therefore initiated a preliminary clinical study in women who had recovered from a previous episode of PPCM and presented with a subsequent pregnancy. Six out of 12 women received BR in addition to standard therapy up to 3 months postdelivery while six patients received standard treatment (peripartum ejection fraction, EF, was similar in both groups; Table S9). In patients receiving BR postdelivery, PRL serum levels, which were elevated more than 5-fold, returned to nonpregnant levels within 14 days of treatment (Figure 5D) as described previously (Harrison, 1979). Three months postpartum, all six

BR-treated women had preserved or increased LV function and dimensions (Table S9) and survived the 4 month observation period. In contrast, the EF in the non-BR-treated (UT) group was deteriorated and three women had died within 4 months (Table S9).

DISCUSSION

We present evidence that pregnancy-related adaptive hypertrophy is associated with enhanced cardiac angiogenesis and that maintenance of the latter in the postpartum phase critically depends on STAT3. The absence of cardiomyocyte STAT3 in the postpartum heart causes increased oxidative stress due to blunted induction of the antioxidant enzyme MnSOD. As a consequence, expression and proteolytic activity of CD are increased, which in turn, induces a detrimental conversion of the nursing hormone PRL into its antiangiogenic 16 kDa derivative. The generation of 16 kDa PRL greatly accelerates the negative effects of oxidative stress and activated CD. In fact, its detrimental effects on the coronary microvasculature promote myocardial hypoxia and apoptosis, thereby contributing to the development of PPCM. Thus, we provide evidence that enhanced activity and release of CD mechanistically connects the increased oxidative stress in STAT3-deficient cardiomyocytes to the development of PPCM. In fact, our study links cardiac processing of PRL into an antiangiogenic and proapoptotic 16 kDa peptide by CD to PPCM and implies that blocking the secretion of PRL can prevent PPCM in mice. Initial clinical observations are consistent with the notion that this beneficial effect of BR may also apply to patients with PPCM.

Enhanced myocardial capillary density is part of the physiological hypertrophy, which the maternal heart undergoes to compensate for the increased volume and workload during pregnancy and labor. Because NT-Prg and CKO-Prg female mice showed increased myocardial capillary density late in pregnancy, this process seems to be independent from cardiomyocyte STAT3. Increased myocardial capillary density is maintained up to 3 weeks postpartum in NT-PP females. In CKO-PP females, however, enhanced myocardial capillary density is lost in the postpartum phase, implying a novel role of STAT3 as a key requisite for maintaining postpartum myocardial angiogenesis. Failure of this process leads to hypoxia as indicated by the increased expression of the hypoxia marker genes HIF1α (Chi and Karliner, 2004) and BNIP3 (Kubasiak et al., 2002), a lower content of energy rich phosphates, apoptosis, and subsequently, heart failure.

Increased hemodynamic load, occurring during pregnancy and labor, may promote cardiac hypoxia and oxidative stress in CKO mice. However, we never observed symptoms or death in CKO females during pregnancy or at delivery, when hemodynamic load culminates. Likewise, increased hemodynamic load induced by aortic constriction did not affect cardiac hypertrophy, function, and mortality in CKO-NP females (Table S10). Cardiomyocyte hypertrophic growth during pregnancy was maintained, suggesting that cardiomyocyte hypertrophy induced by hemodynamic load is not affected in CKO females and therefore does not appear to be the primary trigger for PPCM in CKO mice.

STAT3 and Akt signaling are known to promote hypertrophy and angiogenesis and to exert protection from apoptosis in the heart (Hilfiker-Kleiner et al., 2004a; Jacoby et al., 2003; Liao, 2004; Negoro et al., 2001) and may therefore have overlapping protective functions in the myocardium. Indeed, we observed a late increase in Akt activation in pregnancy in both genotypes, which was followed by a postpartum deactivation (Supplemental Data). In contrast to Akt, cardiac activation of STAT3 was enhanced in NT females late in pregnancy and postpartum, at least in part as consequence of increased peri- and postpartum serum levels of full-length PRL, because we demonstrated that PRL stimulation activated STAT3 in the heart in vivo and in cardiomyocytes in vitro. Taken together, during pregnancy, elevated Akt activation may ensure physiological adaptation of the heart toward pregnancy-related stress, and this might be sufficient to protect CKO-Prg females despite the lack of STAT3. In NT-PP females, activated STAT3 seems to be sufficient to maintain postpartum cardioprotection; in contrast, the lack of both Akt and STAT3 activation in CKO-PP is detrimental, indicating that among several putative mechanisms (including Akt), STAT3 signaling is necessary for protecting the heart from development of PPCM.

Oxidative stress is known to rise during pregnancy, culminating in the last trimester, and is paralleled by an increase in total antioxidant capacity (Toescu et al., 2002). The antioxidant capacity in normal pregnancies peaks in the postpartum phase, suggesting a need for an efficient antioxidant defense mechanism postpartum (Toescu et al., 2002). Circulating levels of oxLDL are markers of oxidative stress in patients (Weinbrenner et al., 2003). In contrast to normal postpartum women, serum levels of oxLDL in patients with PPCM were elevated, indicating enhanced oxidative stress in these patients. Moreover, LVs from CKO-PP mice display an enhanced production of superoxide anions, suggesting that the lack of cardiomyocyte STAT3 impairs important defense mechanisms against postpartum-related oxidative stress. Indeed, it has been shown that MnSOD, a powerful ROS scavenging enzyme, is under the transcriptional control of STAT3 in cardiomyocytes (Negoro et al., 2001). MnSOD plays a crucial role in the antioxidant defense of the heart, because only a 50% decrease in MnSOD protein levels in heterozygous *mnsod*$^{+/-}$ mice is associated with increased oxidative damage and cardiomyocyte death (Van Remmen et al., 2001). Notably, although this reduction of MnSOD protein levels is associated with subtle alterations in cardiomyocyte mitochondrial functions alone, it is not sufficient to induce cardiomyopathy under basal conditions (Van Remmen et al., 2001). Pregnancy/postpartum-associated stress, however, leads to cardiomyopathy in heterozygous *mnsod*$^{+/-}$ females (Figure S10). Thus, a 50% reduction of MnSOD protein appears to be sufficient to cause

peri/postpartum cardiomyopathy. In fact, we observed that LVs from NT-PP mice showed higher levels of MnSOD mRNA and proteins than LVs from CKO-PP mice, indicating a STAT3-dependent transcriptional regulation of MnSOD in the postpartum heart. In line with a lower oxidative defense, CKO-PP LVs displayed enhanced superoxide anion production, which could be prevented with a pharmacological enhancement of MnSOD-like enzyme activity by treatment of CKO mice with MnTBAP. In addition, MnTBAP prevented cardiac dysfunction and postpartum mortality in CKO mice. Thus, STAT3, via inducing MnSOD expression, promotes important defense mechanisms against oxidative stress in the postpartum heart.

The PPCM phenotype of our mouse model showed a degeneration of the cardiac capillary network, which was prevented by treatment with MnTBAP or by blocking the release of PRL by BR. It has been shown that oxidative stress enhances the expression and activation of CD (Corbacho et al., 2002; Roberg and Ollinger, 1998), an enzyme known to cleave full-length 23 kDa PRL into its antiangiogenic 16 kDa form (Lkhider et al., 2004). The 16 kDa PRL is able to dissociate endothelial cell structures, to impair endothelium-dependent vasorelaxation, and to promote apoptosis in endothelial cells and is considered as a physiological inhibitor of tumor growth (Corbacho et al., 2002; Gonzalez et al., 2004; Tabruyn et al., 2003). Large quantities of pituitary PRL are released into the circulation during lactation (Lkhider et al., 2004), and it has been shown that cleavage of PRL into the 16 kDa form by CD takes place in the extracellular compartment of the mammary glands under physiological conditions (Lkhider et al., 2004). Cardiac levels of active CD protein were markedly increased and detected in the interstitium of CKO-PP mice. The observation that active CD released from CD overexpressing hearts in vivo or from cultured cardiomyocytes released in vitro processes 23 kDa PRL into its 16 kDa form together with the finding that PRL cleavage activity could be blocked by a neutralizing CD antibody in CKO-PP LV supernatant ex vivo strongly suggest that CD is mainly responsible for the generation of 16 kDa PRL. In addition, lowering oxidative stress by MnTBAP prevented the upregulation of activated CD in postpartum CKO females. Therefore, we postulate that oxidative stress enhances CD activity and leads to proteolytic cleavage of PRL in the myocardium of CKO-PP females.

Adenoviral expression of the 16 kDa PRL in the heart reduced cardiac capillary density and cardiac function independently of pregnancy and nursing and, indeed, recapitulates aspects of the cardiac phenotype of PPCM. In contrast to its destructive effect on endothelial cells (Corbacho et al., 2002; Gonzalez et al., 2004; Tabruyn et al., 2003), the 16 kDa PRL did not affect cardiomyocyte survival. However, it attenuated cardiomyocyte metabolic activity and may therefore directly affect cardiomyocyte function, an aspect that will be evaluated in future studies.

Myocardial injury by CD overexpression after adenoviral transfection was markedly enhanced in the presence of high systemic PRL levels due to the generation of

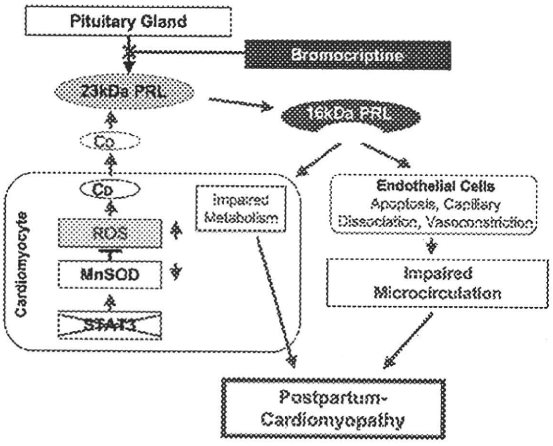

Figure 6. Schematic Model for the Development of PPCM
In the absence of cardiomyocyte STAT3 activity, the postpartum expression of MnSOD is attenuated, leading to increased oxidative stress and the release of CD, which processes 23 kDa PRL into its detrimental 16 kDa form. 16 kDa PRL induces endothelial cell apoptosis, capillary dissociation, and vasoconstriction and impairs cardiomyocyte metabolism, thereby promoting PPCM. Accordingly, BR, a pharmacologic inhibitor of PRL release, prevents PPCM in mice by decreasing circulating PRL.

16 kDa PRL. Moreover, the systemic toxicity of oxidative stress induced by doxorubicin treatment was enhanced in the presence of high systemic PRL levels (Supplemental Data). Thus, the generation of 16 kDa PRL greatly accelerates cardiac injury caused by oxidative stress and CD. Accordingly, the blockade of PRL release by BR prevented the capillary degeneration and the PPCM phenotype in CKO-BR females. Taken together, we propose that the generation of the antiangiogenic 16 kDa PRL by CD-mediated cleavage of the 23 kDa PRL plays a detrimental role for the cardiac microvasculature and, conceivably, for cardiomyocyte metabolism (Figure 6).

In contrast to the MnTBAP treatment, which prevented cardiac capillary degeneration, but not ventricular dilatation, BR provided a more complete rescue of CKO mice from PPCM, including the prevention of ventricular dilatation and the associated upregulation of MMP3. MMP3 has been implicated in ventricular dilatation (Tziakas et al., 2005) and is responsible for a wide range of extracellular matrix degradation and activation of other MMPs (Mukherjee et al., 2005). PRL via activation of STAT5 is able to induce MMP3 expression, a process that can be abolished by BR (Nagafuchi et al., 1999). In fact, attenuated MMP3 expression in CKO-BR mice was associated with a reduction in STAT5 activation, suggesting that a PRL-mediated imbalance of STAT transcription factors in the CKO hearts may, independently from oxidative stress, contribute to the development of PPCM. Further studies will be required to test this hypothesis.

Although STAT3 is markedly reduced in the myocardium from patients with PPCM, our data do not provide information as to whether this reduction of STAT3 is

a primary or a secondary event of PPCM. Nevertheless, similar to our observation in CKO mice, reduced STAT3 may contribute to the development and/or progression of PPCM in patients. So far, sequence analysis of the *stat3* gene in the DNA samples from patients with PPCM and controls did not reveal polymorphisms in the STAT3 gene associated with PPCM (Monika Stoll, personal communication). Our findings of increased CD activity, elevated levels of oxLDL, and 16 kDa PRL in serum samples of nursing PPCM patients, but not in pregnancy-matched controls, point to striking similarities between our PPCM mouse model and human PPCM and are consistent with the notion that oxidative stress, CD activation, and subsequent PRL cleavage may also contribute to the development of PPCM in patients. Our encouraging initial results in a preliminary clinical study with patients treated with BR, a drug widely used to stop lactation in postpartum women (Harrison, 1979), are consistent with the hypothesis that PRL and mainly the 16 kDa PRL are causally related to the development of PPCM in patients. As a limitation of this study, it should be noted that not all effects of BR on its targets were analyzed and that the inhibition of PRL secretion is the most plausible reason for the protective effect of BR in PPCM.

In summary, we show that STAT3 plays a critical role for the preservation of postpartum myocardial angiogenesis and function and suggest a central role of STAT3 and associated signaling pathways in the onset of PPCM (Figure 6). Importantly, we present evidence for a novel mechanism for the development of cardiomyopathy, which involves the cleavage of PRL into its detrimental 16 kDa form by CD (Figure 6). BR, a pharmacological inhibitor of PRL release, prevents PPCM in mice (Figure 6). In consequence, based on our experimental and initial clinical findings, BR may represent a novel therapeutic option to treat patients with PPCM or to prevent the disease in patients who suffered and recovered from PPCM in a previous pregnancy.

EXPERIMENTAL PROCEDURES

PRL (sheep), BR, and all other chemicals were purchased from Sigma.

Cardiomyocyte-Specific Deletion of *stat3*

The generation of mice with cardiomyocyte-restricted deletion of *stat3* has been described (Hilfiker-Kleiner et al., 2004a). Female mice were first bred at the age of 10–12 weeks, at an age when STAT3 protein was barely detectable in isolated cardiomyocytes (Figure S2), indicating that, as in CKO males (Hilfiker-Kleiner et al., 2004a), the Cre-mediated deletion of *stat3* was virtually complete.

Animal Experiments

Generally, analyses were performed 3 weeks after the second delivery. Prolactin (400 iU/kg) was injected i.v. For chronic administration, osmotic minipumps (Alzet; PRL 400 iU/kg/d) were implanted in sedated mice. Adenoviruses, 16K-Ad (Pan et al., 2004), *lacZ*-Ad, or CD-Ad (3×10^8 pfu of each virus) was injected directly into the mouse LV (Supplemental Experimental Procedures). BR (4 mg/kg/d, Novartis) was added in drinking water. MnTBAB (300 μg/mouse/day) or vehicle (placebo) was injected subcutaneously. Transthoracic echocardiography was performed in sedated mice as previously described (Hilfiker-Kleiner et al., 2004a). Hemadynamic measurements were assessed by telemetry (Pelat et al., 2003) or tail cuff in conscious mice or by Millar catheter in anesthetized mice as described previously (Hilfiker-Kleiner et al., 2004a, 2004b). For more detailed description of animal experiments, see the Supplemental Experimental Procedures.

All animal studies were in compliance with the *Guide for the Care and Use of Laboratory Animals* as published by the U.S. National Institutes of Health and were approved by our local Institutional Review Boards.

Patients Data

LV samples were from patients undergoing heart transplantation due to PPCM (NYHA functional class III or IV) and from donor hearts (NF) that could not be transplanted for technical reasons. Serum was obtained from PPCM patients (NYHA functional class III or IV) at their first presentation and from age and pregnancy-matched nursing healthy woman. oxLDL was determined with a sandwich ELISA kit (Mercodia). More detailed patient data are presented in the Supplemental Experimental Procedures.

Histological Analyses and Immunostaining

For histological analyses, hearts were fixed in situ, embedded in paraffin, and stained with picro-Sirus red or H&E, as described (Hilfiker-Kleiner et al., 2004a). Interstitial collagen volume fraction was determined in picro-Sirius red-stained sections as described (Hilfiker-Kleiner et al., 2004a). Apoptotic nuclei were detected by in situ terminal deoxynucleotidyl transferase-mediated digoxigenin-conjugated dUTP nick end labeling (TUNEL) and by nuclear morphology using Hoechst 33258 staining (Hilfiker-Kleiner et al., 2004a).

Capillary Density

Capillary density was determined as the ratio of capillaries to 100 cardiomyocytes in transversely sectioned LV tissue immunostained with isolectin B4 (Vector) or the platelet-endothelial cell adhesion molecule-1 (PECAM-1) antibody (Santa Cruz) and counterstained with WGA and Hoechst 33258 as described previously (Hilfiker-Kleiner et al., 2004a).

Measurement of Myocardial Superoxide Production and NADH activity

Dihydroethidium fluorescence staining was used for in situ detection of superoxide production as described (Engberding et al., 2004). NADH activity was determined in LV myocardium (50 μg protein) by electron spin resonance (ESR) spectroscopy as described previously (Spiekermann et al., 2003). Both methods are described in the Supplemental Experimental Procedures.

Measurement of Energy-Rich Phosphates in Myocardial Tissue

AMP, ADP, and ATP were measured as described previously (Hilfiker-Kleiner et al., 2004a). A brief description is provided in the Supplemental Experimental Procedures.

CD Activity Assay

Supernatant from freshly isolated LV tissue was generated by mincing LV tissue in ice-cold HBSS. Sedimented minced LV tissue was then incubated in DMEM for 1 hr at 37°C and 5% CO_2. CD activity was determined in LV supernatant or in patient serum by using the InnoZyme CD Immunocapture Activity Assay Kit (Calbiochem) and a FLUOstar Galaxy.

PRL Cleavage Assay

PRL cleavage activity was assayed in LV supernatant (described above) by adding citrate/phosphate buffer (pH 3.5) at a 1:1 ratio; subsequently, pepstatin A (2 mg/L) and/or recombinant PRL (0.1 g/L) was added and mixed and incubated at 37°C (5% CO_2) for 2 hr. After addition of an equal volume of Laemmli buffer (5% BME, 0.2% DTT),