Universitat de Lleida

Developmental switch on the signaling for cardiomyocyte death: Insights into the regulation of apoptotic gene expression and into the control of DNA fragmentation

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Daniel Sanchis Morales, Doctor en Ciències Biològiques, investigador *Ramon y Cajal* al Departament de Ciències Mèdiques Bàsiques de la Universitat de Lleida i director de la present tesi,

Faig constar que,

El llicenciat en Medicina per *Shanxi Medical University (Xina)* i Màster en Bioquímica per *Fudan University (Xina)*, **Jisheng Zhang**, ha realitzat sota la meva direcció i supervisió dins del grup de Senyalització Cel·lular i Apoptosi del Departament de Ciències Mèdiques Bàsiques, el treball experimental titulat "*Developmental switch on the signaling for cardiomyocyte death: Insights into the regulation of apoptotic gene expression and into the control of DNA fragmentation*".

El treball reuneix les condicions adients per tal de poder ser defensat davant del Tribunal de Tesi corresponent i, si s'escau, obtenir el grau de **Doctor** per la Universitat de Lleida.

I perquè així consti i als efectes oportuns signo el present document a

Lleida, 15 de setembre de 2009

Dr. Daniel Sanchis Morales

献给我亲爱的父母

和兄长

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Jisheng Zhang, 张继生

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Abbreviations

AIF	Apoptosis Inducing Factor
APAF-1	Apoptosis Protease Activation Factor 1
ANT	Adenine Nucleotide Translocator
BH	Bcl-2 homology domain
CARD	Caspase Recruitment Domain
ced	Cell death abnormality
CRD	Cystein Rich Domain
DD	Death Domain
DED	Death Effector Domain
DISC	Death Inducing Signaling Complex
DNA	Deoxyribonucleic Acid
DR	Death Receptor
EndoG	Endonuclease G
FADD	Fas Associated Death Domain
FLICE	FADD-like Interleukin-1β-converting enzyme
FLIP	Fas-Associated Death Domain-like Interleukin-1-beta-converting enzyme-inhibitory protein
hnRNP	Heterogeneous Nuclear Ribonucleoproteins
IAP	Inhibitor of Apoptosis Protein
IBM	IAP Binding Motifs
ICE	Interleukin-1b-converting Enzyme
IKK	Inhibitor of κB Kinase
IMM	Inner Mitochondrial Membrane
IRES	Internal Ribosome Entry Site
JNK	c-jun N-terminal Kinase

kDa	kiloDalton
MEF	Mouse Embryonic Fibroblast
MOMP	Mitochondrial Outer Membrane Permeabilization
MPTP	Mitochondrial Permeability Transition Pore
NF-κB	Nuclear Factor Kappa B
OMM	Outer Mitochondrial Membrane
PARP	Poly-ADP Ribose Polymerase
PCD	Programmed Cell Death
PTB	Polypyrimidine tract binding protein
RIP	Receptor Interacting Protein
RNA	Ribonucleic Acid
ROS	Reactive Oxygen Species
RRM	RNA Recognition Motif
SMAC	Second Mitochondria-Derived Activator of Caspase
shRNA	Short Hairpin RNA
tBID	Truncated Bid
TNF	Tumor Necrosis Factor
TRADD	TNFR-Associated Death Domain
TRAF	TNFR-Associated Factor
TRAIL	TNF-Related Apoptosis Inducing Ligand
TUNEL	Terminal deoxynucleotidyl Transferase Biotin-dUTP Nick End Labeling
UTR	Untranslated Region
VDAC	Voltage-Dependent Anion Channel

Abstract

L'apoptosi s'ha considerat un factor important en el control del balanç entre proliferació cel·lular, diferenciació i mort durant el desenvolupament. La regulació de l'apoptosi és un factor clau per aconseguir aquest balanç, eliminant cèl·lules innecessàries o malmeses que, d'altra manera, podrien tenir efectes adversos per l'organisme. Durant la darrera dècada, molts treballs han demostrat que la senyalització per apoptosi té una funció vital per la morfogènesi del cor. Malgrat això, existeix controvèrsia sobre la implicació de les caspases en la mort dels cardiomiòcits en l'individu adult. Així, l'anàlisi de la regulació de l'apoptosi des d'una perspectiva de desenvolupament podria millorar el nostre coneixement sobre la funció de l'apoptosi al cor. Per tant, els nostres principals objectius han estat caracteritzar la regulació de l'expressió dels gens apoptòtics durant el desenvolupament cardíac i la identificació de les vies alternatives implicades al dany del teixit cardíac.

Vàrem analitzar l'expressió de proteïnes implicades al control de la via de mort cel·lular dependent de caspases en teixits provinents de rates de diferents edats entre l'embrió i l'estadi adult. Els gens implicats en l'activació de l'apoptosi es troben fortament silenciats al cor adult i, la major part d'ells, també al cervell, però no al fetge. Les excepcions varen ser Caspasa-2, l'expressió de la qual es reprimeix a tots els teixits estudiats durant la fase embrional, i Bax i Bak, que es silencien en tots els teixits durant el període perinatal. L'expressió de la caspasa iniciadora Caspasa-9 i les caspases executores Caspasa-3 i Caspasa-7 es troba reprimida al cor i cervell postnatals. En relació amb els gens anti-apoptòtics, Bcl-2 es silencia al cor i el cervell durant el desenvolupament i no es va detectar al fetge, mentre que Bcl-XL s'expressa a tots els teixits fins l'estadi adult. Mitjançant l'anàlisi per RT-PCR semi-quantitativa vàrem poder observar que la reducció en l'abundància de les proteïnes apoptòtiques durant el desenvolupament té lloc de forma paral·lela amb la reducció en els nivells dels seus mRNA. Durant la isquèmia experimental o el tractament amb la droga pro-apoptòtica estaurosporina, l'activació de la caspasa executora Caspasa-3 va observar-se només als cardiomiòcits embrionaris, però no als cardiomiòcits postnatals o adults. A més a més, la isquèmia va induir fragmentació de l'ADN que no es va poder evitar afegint inhibidors de caspases a cardiomiòcits postnatals en cultiu, que ja expressen nivells molt baixos d'aquests gens.

Per continuar amb la investigació sobre la mort dels cardiomiòcits postnatals i el canvi d'una via dependent de caspases a una via independent de caspases durant el desenvolupament del cor, vàrem analitzar el possible rol de diverses DNAses que podrien estar implicades al dany a l'ADN durant la isquèmia. La repressió experimental en cultiu del gen Endonucleasa G (EndoG), que codifica per una nucleasa mitocondrial, va bloquejar la degradació de l'ADN durant la isquèmia, demostrant que EndoG té una funció molt important en aquest esdeveniment, de forma independent a les caspases. La sortida d'EndoG del mitocondri és un pas important per a la fragmentació de l'ADN als cardiomiòcits. Hem demostrat que Bcl-XL és capaç de bloquejar i Bnip3 contribueix a l'alliberament d'EndoG del mitocondri i, així, tenen efectes contraposats en la fragmentació de l'ADN durant la isquèmia als cardiomiòcits diferenciats.

També vàrem iniciar l'anàlisi dels mecanismes que controlen l'expressió i silenciament dels gens apoptòtics al cor. Vàrem analitzar la contribució de dos candidats, E2F i les proteïnes d'unió a tractes de polipirimidines (PTB). Al cor, l'expressió d'E2F i PTB es redueix durant el desenvolupament, de forma paral·lela als gens apoptòtics. Malgrat això, l'anàlisi de l'expressió de gens apoptòtics a ratolins deficients per E2F1, E2F2 o tots dos gens no va mostrar cap canvi en relació amb els nivells d'expressió als seus respectius animals control ni durant l'estadi embrional ni durant el desenvolupament postnatal. Aquests resultats varen descartar que E2F tinguin una funció rellevant en el control de l'expressió dels gens apoptòtics al cor, a diferència del que s'ha observat en línies cel·lulars tumorals.

D'altra banda, vàrem trobar que, en cardiomiòcits neonatals, que expressen nivells molt baixos de PTB, la sobrexpressió de PTB1 o PTB4 va induir la re-expressió de molts gens apoptòtics, incloent Apaf-1, les caspases-9 i -3, i Bid. A més a més, els cardiomiòcits sobre-expressors de PTB varen patir major dany a l'ADN durant la isquèmia que els cardiomiòcits control i aquest increment podia ser inhibit mitjançant inhibidors de caspases fins a nivells similars als de cèl·lules no sobre-expressores. Això suggereix que la recuperació de l'expressió de PTB, probablement implicant l'increment de l'expressió de gens apoptòtics, va induir un canvi a un mecanisme implicant les caspases. La re-expressió de PTB va induir un increment en

la quantitat de proteïnes apoptòtiques sense afectar els nivells dels seus respectius mRNA. Els assaigs luciferasa (Luc) en cardiomiòcits normals i sobre-expressors de PTB, utilitzant plàsmids bicistrònics en els quals es va integrar la regió 5'-no codificant (5'-UTR) dels transcrits d'Apaf-1 o Caspasa-3 entre LucRenilla i LucFirefly, varen demostrar un increment en l'activitat LucFirefly quan qualsevol de les dues 5'-UTR es trobava present, comparat amb els nivells control. Aquests resultats suggereixen que PTB indueixen traducció per la via Internal Ribosolmal Entry Site (IRES), que requereix certes seqüències a les regions 5'-UTR dels gens implicats.

La apoptosis se ha considerado un factor importante en el control del balance entre proliferación celular, diferenciación y muerte durante el desarrollo. La regulación de la apoptosis es un factor clave para conseguir este balance, eliminando células innecesarias o dañadas que, de otro modo, podrían tener efectos adversos para el organismo. Durante la última década, muchos trabajos han demostrado que la señalización por apoptosis desempeña una función vital para la morfogénesis del corazón. Sin embargo, existe controversia sobre la implicación de las caspasas en la muerte de los cardiomiocitos en el individuo adulto. Así, el análisis de la regulación de la apoptosis desde una perspectiva del desarrollo, podría mejorar nuestro conocimiento sobre la función de la apoptosis en el corazón. Por tanto, nuestros principales objetivos fueron caracterizar la regulación de la expresión de los genes apoptóticos durante el desarrollo cardiaco y la identificación de vías alternativas implicadas en el daño del tejido cardiaco.

Analizamos la expresión de proteínas implicadas en el control de la vía de muerte celular dependiente de caspasas en tejidos provenientes de ratas de diferentes edades entre el embrión y el estadio adulto. Los genes implicados en la activación de la apoptosis se encuentran intensamente silenciados en el corazón adulto y, la mayor parte de ellos, también en el cerebro, pero no en el hígado. Las excepciones fueron Caspasa-2, que se reprime en todos los tejidos en período embrional, y Bax y Bak, que se silencian en todos los tejidos durante el desarrollo perinatal. La expresión de la caspasa iniciadora Caspasa-9 y de las caspasas ejecutoras -3 y -7 se encontró reprimida en el corazón y cerebro postnatales. En relación con los genes antiapoptóticos, Bcl-2 se silencia en el corazón y el cerebro durante el desarrollo y no se detectó en hígado, mientras que Bcl-XL se expresa en todos los tejidos hasta el estadio adulto. Mediante el análisis por RT-PCR semi-cuantitativa pudimos observar que la reducción de la cantidad de proteínas apoptóticas durante el desarrollo ocurría en paralelo con una reducción en el nivel de sus mRNA. Durante la isquemia experimental o el tratamiento con la droga pro-apoptótica estaurosporina, la activación de la caspasa ejecutora Caspasa-3 se observó solamente en cardiomiocitos embrionarios, pero no en cardiomiocitos postnatales o adultos. Además, la isquemia indujo fragmentación del ADN que no se pudo frenar con inhibidores de caspasas en

cardiomiocitos postnatales, que ya expresan niveles muy bajos de los genes apoptóticos.

Para continuar con la investigación sobre la muerte de cardiomiocitos postnatales y el cambio de una vía dependiente de caspasas a una vía independiente de caspasas durante el desarrollo del corazón, analizamos el posible rol de diversas DNAsas que podían estar implicadas en el daño al ADN durante la isquemia. La represión experimental en cultivo del gen Endonucleasa G (EndoG), que codifica para una nucleasa mitocondrial, bloqueó la degradación del ADN durante la isquemia, demostrando que EndoG tiene una función importante en la fragmentación del ADN durante la isquemia, de forma independiente a la activación de caspasas. La salida de EndoG de la mitocondria es un paso importante para la fragmentación del ADN en cardiomiocitos. Demostramos que Bcl-XL es capaz de bloquear y BNip3 contribuye a la salida de EndoG de la mitocondria y, así, tienen efectos contrapuestos en la fragmentación del ADN durante la isquemia en cardiomiocitos diferenciados.

También iniciamos el análisis de los mecanismos que controlan la expresión y silenciamiento de la expresión de los genes apoptóticos en el corazón. Analizamos dos candidatos, E2F y las proteínas de unión a tractos de polipirimidinas (PTB). En el corazón, la expresión de E2F y PTB se reduce durante el desarrollo, en paralelo a los genes apoptóticos. Sin embargo, el análisis de la expresión de genes apoptóticos en ratones deficientes para E2F1, E2F2 o ambos genes no mostró ningún cambio en relación con sus respectivos ratones controles ni durante el estadio embrional ni durante el desarrollo postnatal. Estos resultados descartaron que E2F tenga una función relevante en el control de la expresión de genes apoptóticos en el corazón, a diferencia de lo observado en líneas celulares tumorales.

Por otro lado, encontramos que en cardiomiocitos neonatales, que expresan niveles muy bajos de PTB, la sobre-expresión de PTB1 o PTB4 indujo la re-expresión de muchos de los genes apoptóticos, incluyendo Apaf-1, las caspasas 9 y 3 y Bid. Además, los cardiomiocitos sobre-expresores de PTB sufrieron más daño al ADN durante la isquemia que los cardiomiocitos control y este incremento podía ser inhibido mediante inhibidores de caspasas a niveles similares a los de células no

sobre-expresoras. Esto sugiere que la recuperación de la expresión de PTB, probablemente implicando el incremento de expresión de genes apoptóticos, indujo el cambio a un mecanismo dependiente de caspasas en los cardiomiocitos. La re-expresión de PTB indujo un incremento en la cantidad de proteínas apoptóticas sin afectar los niveles de sus respectivos mRNA. Los ensayos luciferasa (Luc) en cardiomiocitos normales y sobre-expresores de PTB, utilizando plásmidos bicistrónicos en los cuales se integró la región 5'-no codificante 5'UTR de los transcritos de Apaf-1 o Caspasa-3 entre LucRenilla y LucFirefly, demostraron un incremento en la actividad LucFirefly cuando cualquiera de las 5'UTR se encontraba presente, comparado con los niveles control. Estos resultados sugieren que PTB inducen traducción por vía Internal Ribosome Entry Site (IRES), que requiere de determinadas secuencias en las regiones 5'UTR de los genes implicados.

Abstract

Apoptosis has been considered as an important factor in controlling the balance between cell proliferation, differentiation and death during development. The regulation of apoptosis is a key aspect to achieve this balance, by eliminating unnecessary or miss-specified cells which, otherwise, may have harmful effects on the whole organism. In the past decade, extensive observations demonstrated that apoptosis plays a vital role in heart morphogenesis. However it is still controversial whether Caspases are involved in apoptosis of cardiomyocytes in the adult. Thus, the analysis of apoptosis regulation from a developmental perspective could improve our knowledge about how apoptosis acts in the heart. Therefore, our main objectives were to characterize the regulation of the expression of apoptotic genes during heart development and to identify alternative pathways involved in cardiac damage.

Expression of proteins involved in the control of Caspase-dependent cell death was analyzed in tissues from rats of different ages ranging from the embryo to the adult. The genes involved in the induction of cell death were intensely repressed in the adult heart and, most of them, also in the brain but not in the liver. Exceptions were Caspase-2, which was only expressed in embryos, and Bak and Bax, which were downregulated in all three tissues during postnatal development. The expression of Caspase-9 and executioner Caspase-3 and -7 was reduced in postnatal heart and brain. With regard to the expression of anti-apoptotic proteins, Bcl-2 was silenced in heart and brain during development and undetectable in the liver, whereas Bcl-XL was expressed in all tissues until adulthood. Using the semi-quantitative RT-PCR method, we found that apoptotic gene silencing involved a reduction of the transcript amount for these genes. During ischemia or staurosporine treatment executioner Caspase-3 activation was only observed in embryonic cardiomyocytes, but not in postnatal and adult cardiomyocytes. Furthermore, ischemia induced DNA fragmentation that was not inhibited by Caspase inhibitors in neonatal cardiomyocytes, which already express very low levels of apoptotic genes.

To continue the investigation of cell death in postnatal cardiomyocytes and the switch from Caspase-dependent to Caspase-independent pathway during heart development, we assessed the potential role of several DNAse which may be involved in ischemia-induced DNA damage. Knockdown of mitochondrial nuclease

gene Endonuclease G (EndoG) decreased the ischemia induced DNA damage, which demonstrated that EndoG plays an important role to the ischemia induced DNA fragmentation in this Caspase-independent cell death model. EndoG release from mitochondria is the key step to DNA fragmentation. We have shown that Bcl-XL hampers and BNip3 contributes to the release process of EndoG and, thus, affect in opposite ways to ischemia induced DNA damage in differentiated cardiomyocytes.

The mechanisms controlling the repression of major apoptotic gene expression have also been investigated. We analyzed two potential candidates, E2F and Polypyrimidine tract binding proteins (PTB). In the heart, the expression of E2F and PTB decrease during development, in a similar way to the decrease of apoptotic gene expression. But the analysis of E2F1 and E2F2 single and double knockout mice showed no differences neither in the embryonic expression of these genes nor in their repression during development when compared with their wild type littermates. These results discarded a relevant role of E2F in the regulation of apoptotic gene expression during heart development, contrary to the observations in tumor cell lines.

On the other hand, we found that in neonatal cardiomyocytes which express low levels of PTB overexpression of PTB1 and PTB4 induced expression of most of the apoptotic proteins, including Apaf-1, Caspase-9, Caspase-3 and Bid. In addition, neonatal cardiomyocytes overexpressing PTBs had more DNA damage during ischemia and the DNA fragmentation was reduced to the level of “wild type” cells by Caspase inhibitors. This suggested that with recovery of PTB expression level, likely involving the PTB-dependent restoring of Caspase-dependent mechanisms, DNA damage can go back to the Caspase-dependent mechanism in cardiomyocytes. PTB induced an increase in the amount of apoptotic proteins without affecting their transcripts. Luciferase (Luc) assays in wild type and PTB overexpressing cultured cardiomyocytes, using bicistronic plasmids in which the 5’UTR of Caspase-3 and Apaf-1 were located between Luc Renilla and Luc Firefly, showed an increase of Luc Firefly activity when preceded by either 5’UTR, compared with the control levels. These results suggested that PTBs upregulate apoptotic gene expression at the post transcriptional level via IRES-dependent translation.

摘要

发育依赖于细胞增殖、分化和死亡的相互平衡，细胞凋亡在调控这一平衡中起重要作用。通过凋亡多余的细胞或者非特异细胞被清除，细胞凋亡的失控将打破这一平衡，无疑会对机体造成极大伤害。另外对细胞凋亡本身的调控是控制此平衡的一个重要方面。在过去的几十年中，很多证据表明细胞凋亡在心脏形成中起重要作用，但是 Caspases 是否参与了成体心肌细胞凋亡尚有争论。因此从发育的角度对细胞凋亡调控的研究将有助于提高对细胞凋亡在心脏中如何发挥作用的认识。我们的主要目标是确定在心脏发育中凋亡基因表达如何调控， 同时确定在心肌损伤中是否存在别的通路。

首先我们检测了从胚胎到成体不同发育时期大鼠心脏、大脑和肝脏组织中参与 Caspase 依赖性细胞凋亡蛋白的表达水平。我们发现大部分调控细胞凋亡的蛋白表达在成体大鼠心脏受到了强烈的抑制。其中 Caspase-2 仅在胚胎表达，Bak 和 Bax 在三种组织中蛋白表达都受到抑制；除此之外其余大部分蛋白在大脑的表达也同样受到了抑制，但是在肝脏并未有明显下调。Caspase-9 和效应 Caspase-3 和-7 在心脏和大脑表达下降。抗凋亡蛋白因子中，Bcl-2 在心脏和大脑发育过程中被抑制，而在肝脏中未检测到 bcl-2 表达；Bcl-XL 在三种组织中蛋白表达未观察到明显抑制，在成体组织中仍有相当程度的表达。使用半定量 RT-PCR 方法，我们发现这些凋亡基因在转录水平也受到了抑制，和蛋白表达水平的抑制相似。缺氧或者星形孢菌素（staurosporine）处理后，效应 Caspase-3 的激活仅在胚胎心肌细胞可以被检测到，而在新生鼠或者成体大鼠心肌细胞则不能被检测到，并且缺氧引起的低表达凋亡基因的新生鼠心肌细胞 DNA 断裂不被 Caspase 抑制剂所抑制。

以上结果表明，在出生后大鼠心肌细胞凋亡通路已经由 Caspase 依赖性转化为 Caspase 非依赖性。为进一步研究这一转换机制和对生后心肌细胞死亡，我们对可能参与缺氧导致的 DNA 断裂的一些 DNA 酶的作用进行了检测。抑制位于线粒体的脱氧核糖核酸内切酶 EndoG 可以下调缺氧引起的 DNA 断裂，这表明 Caspase 非依赖性心肌细胞凋亡中 EndoG 对缺氧引起的 DNA 断裂发挥重要作用。EndoG 从线粒体释放是一个关键步骤。我们的实验表明 Bcl-XL 阻碍 EndoG 从线粒体释放到细胞浆，而 BNip3 促进 EndoG 的释放；Bcl-XL 和 BNip3 在缺氧引起的心肌细胞 DNA 断裂中发挥相反的作用。

同时我们也对控制主要凋亡基因表达抑制的机制进行了研究。我们检测了两个极有可能的蛋白，E2F 和多嘧啶序列结合蛋白（PTB）。在心脏发育中 E2F 和 PTB 表达下降，这和凋亡基因表达下降相似。但是对 E2F1、E2F2 和 E2F1/E2F2 双基因敲除小鼠的的研究表明，基因敲除小鼠无论在胚胎表达还是发育中表达的抑制和野生型小鼠比较都没有差别。和在肿瘤细胞系中的观察到的 E2F 的作用相反，我们的结果表明在心脏发育过程中 E2F 对凋亡基因表达的调控没有重要作用。

另一方面，我们发现在 PTB 表达很低的新生鼠心肌细胞中过表达 PTB1 和 PTB4 可以上调包括 Apaf-1、Caspase-9、Caspase-3 和 Bid 的大部分凋亡分子蛋白表达。而且过表达 PTB 的新生鼠心肌细胞和对照相比在缺氧时有更多的 DNA 分子断裂， 并且在 Caspase 抑制剂的作用下高水平的 DNA 断裂可以恢复到对照水平。这一结果提示过表达后 PTB 表达水平恢复可能导致了新生鼠心肌细胞 DNA 断裂从 Caspase 非依赖性回到 Caspase 依赖性的细胞凋亡机制，这一逆转换是依赖于 PTB 的。 过表达 PTB 引起的凋亡蛋白水平上调但并未影响其转录水平。克隆 Caspase-3 和 Apaf-1 的 5’非翻译区于 Renilla 和 Firefly 之间，构建荧光素酶报告基因实验所需的双顺反子表达载体。实验显示和对照相比 PTB 过表达的新生鼠心肌细胞中 Firefly 的活性上升。这些结果表明 PTB 上调凋亡基因表达位于转录后水平，是内部核糖体进入位点序列（IRES）依赖的蛋白翻译。

Introduction

1. APOPTOSIS

1.1 Apoptosis: historical background

The term 'apoptosis' was firstly used by Kerr, Wyllie and Currie in 1972 to describe a mode of cell death (Kerr et al., 1972). This cell biology phenomenon was initially clarified in the *Caenorhabditis elegans***.** Sulston and Horvitz found that 131 out of 1090 somatic cell invariantly undergo programmed cell death during development of *C. elegans* (Sulston and Horvitz, 1977; Sulston et al., 1983). In 1990s some molecular regulators were found to control this developmental cell death. The central components of the programmed cell death in *C. elegans* are three CED (cell death abnormal), namely CED-3, CED-4 and CED-9. In mammals, the homologs of CED-9, CED-4 and CED-3 are present in the Bcl-2 family, Apaf-1/NOD-like receptor family and Caspase (cysteinyl aspartate proteinases) family, respectively (Li and Yuan, 2008). Apoptotic cell death is a strictly controlled process in which every single step is highly regulated by these proteins whose function is to carry on the whole suicide program (Shi, 2002).

Apoptosis or programmed cell death were termed and used historically. Our concept to this type of cell death has been extended with the development of investigation. According to classification of the Nomenclature Committee on Cell Death (NCCD), the NCCD decided that the 'official' classification of cell death modalities had to rely on purely morphological criteria, owing to the absence of a clear-cut equivalence between ultrastructural alterations and biochemical cell death characteristics. The morphological changes that define the best-studied modality of cell death, apoptosis (type 1 cell death), include nuclear pyknosis (chromatin condensation) and karyorhexis (nuclear fragmentation). Apoptotic cells finally form small round bodies that are surrounded by membranes and contain intact cytoplasmic organelles or fragments of the nucleus. These apoptotic bodies result from progressive cellular condensation and budding, and eventually are engulfed by resident phagocytic cells (e.g. epithelial cells or fibroblasts) (Galluzzi et al., 2007).

Apoptosis can be characterized difficultly with biochemical criteria because this type of cell death can occur without oligonucleosomal DNA fragmentation as well as without Caspase activation. Initially, apoptosis and Caspase-dependent cell death were used as equivalent terms. It is true that the contribution of Caspases is

frequently observed in apoptosis, but noncaspase death effectors (e.g. AIF, EndoG and HtrA2/Omi) as well as the deleterious consequences of failing mitochondrial metabolism also can lead to cell death even when effector Caspases are inhibited (Kroemer and Martin, 2005). Thus, although apoptosis often occurs with Caspase activation and the acquisition of some of the hallmarks of apoptosis is strongly Caspase-dependent, apoptotic death does not occur entirely through Caspase activation.

We can not oversimplify the definition of apoptosis. For example, cell death that usually manifests with an apoptotic morphology can be shifted to a more necrotic phenotype when Caspase activation is prevented by pharmacological inhibitors or by the elimination of essential Caspase activators such as Apaf-1 (Golstein and Kroemer 2005; Kroemer and Martin 2005). Therefore, we need to understand comprehensively the morphological and biochemical processes in apoptosis taking into account each particular paradigm.

1.2 Caspases

1.2.1 Caspase family and functional classification

Caspases (***c***ysteinyl ***asp***artate protein***ases***) are evolutionarily conserved cysteine proteases expressed as inactive zymogens in virtually all animal cells. When activated, these enzymes cleave their substrates on the carboxy-terminal side of an aspartate residue. So far, Caspase gene family contains 14 mammalian members, of which, 11 human enzymes are known including Caspase-1 to Caspase-10 and Caspase-14. And 10 genes were found in the mouse genome to encode 10 murine Caspases, Caspase-1, 2, 3, 6, 7, 8, 9, 11, 12 and 14. The human Caspase-4 and 5 are functional orthologs of mouse Caspase-11 and 12, whereas human Caspase-10 is absent in the mouse genome. The remaining Caspases are functional orthologs between the human and mouse (Li and Yuan, 2008; Zimmermann et al., 2001).

Caspases can be defined according to their structure (Figure 1). Initiator Caspases contain protein-protein interaction motifs: the death effector domain (DED) (Caspase-8,10) or the Caspase recruitment domain (CARD) (Caspase-1,2,4,5,9,11,12) which interact with the upstream adaptor molecules, whereas the

effector Caspases (Caspase-3, -6, -7) with short prodomains perform downstream execution steps of apoptosis by cleaving multiple cellular substrates and are typically processed and activated by upstream Caspases (Li and Yuan, 2008). Different from other short domain Caspases, the expression of Caspase-14 is confined to the epithelium and it is involved in keratinocyte differentiation (Denecker et al., 2008).

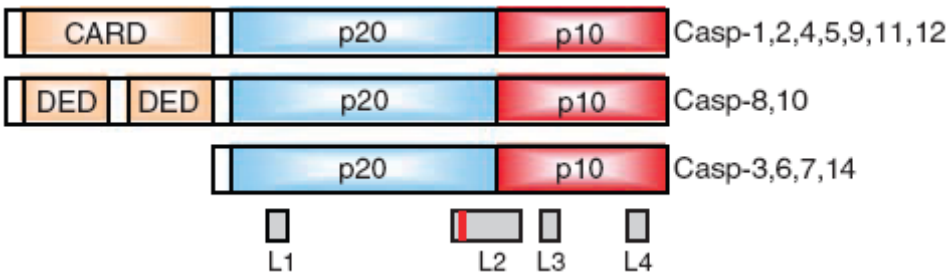


Figure 1. Structure and domain organization of mammalian Caspases: Domain organization of Caspases and the location of catalytic center loops (L1–L4). Initiator Caspases have long prodomains, CARD or DED, whereas executioner Caspases have short prodomains. Loops are shown in gray. The active site Cys is shown by a red line. Processing that separates p20 and p10 subunits occur in L2. The resulting large subunit potion of the L2 loop of one monomer and small subunit portion of the L2 loop of the neighboring monomer (L20) are involved in loop bundle formation (Li and Yuan, 2008).

Caspases are synthesized as inactive proenzymes, which are activated following cleavage at specific aspartate cleavage sites. Phylogenetic analysis of the Caspases reveals that there are three subfamilies: an ICE subfamily, comprising Caspase-1, -4 and -5, a CED-3}CPP32 (32 kDa cysteine protease) subfamily, comprising Caspase-3, -6, -7, -8, -9 and -10, and an ICH-1 (where ICH is *Ice* and *ced-3* homologue)}Nedd2 subfamily (Cohen, 1997) (Figure 2).

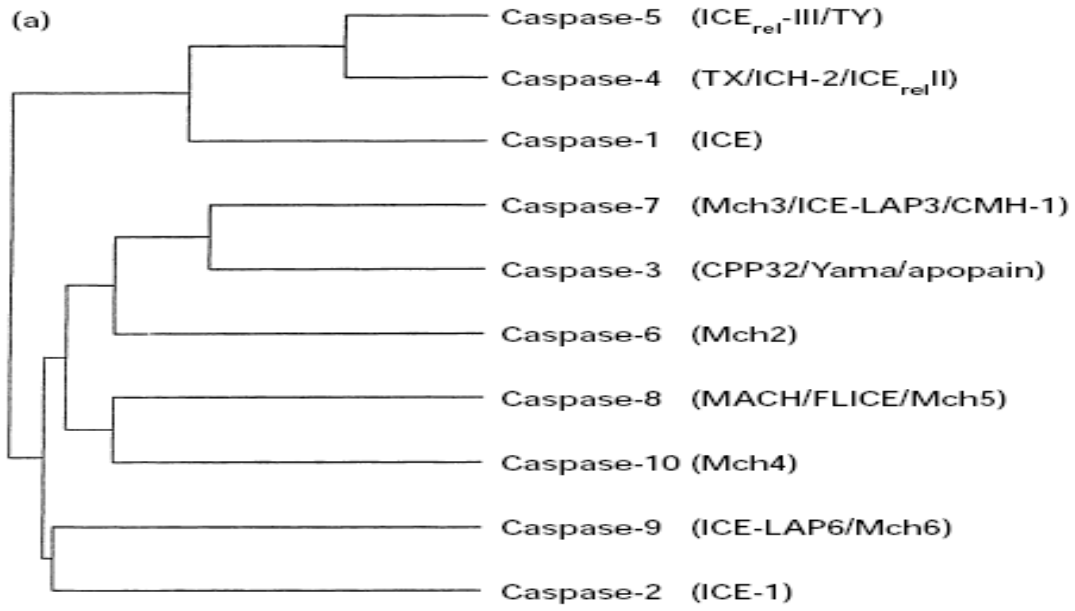


Figure 2. Phylogenetic relationships. Phylogenetic relationships were determined and polypeptide sequences for the human forms of the Caspases aligned using the PILEUP algorithm (Genetics Computer Group, Madison, WI, U.S.A.). The relationships are based on the full-length proenzymes, which is appropriate as the prodomains clearly play a functional role (Cohen, 1997).

1.2.2 Caspase activation

Caspases are produced in inactive form containing a prodomain (only for initiator Caspases), a large subunit p20 and a small subunit p10. Caspases are activated by two cleavage events. The p20 subunit consists of the active site Cys which is a part of conserved QACXG motif. The first proteolytic cleavage divides the chain into large and small Caspase subunits and the second cleavage removes the N-terminal prodomain. The activated Caspase is a tetramer of two large and two small subunits, with two active sites (Wolf and Green, 1999). To clarify this process we can distinguish into 2 different mechanisms (Figure 3).

For initiator Caspases the prodomains vary in length and sequence which help Caspases function as an adaptor for pro-inflammatory or apoptotic signal. To be activated, i.e. Caspase-8, it requires the assembly of a multi-component complex formed upon exposure to death stimuli. This results in the recruitment of high concentrations of procaspase-8 which, although presenting low protease activity, are sufficient to induce its own cleavage and activation by protein-protein interactions (Riedl and Shi, 2004). In a word the initiator Caspases are auto-activated.

Another case is procaspase-9 activation which needs the apoptosome complex. The Apaf-1 oligomerizes to form a wheel-shaped signaling platform with cytochrome C released from mitochondria and other cofactors such as dATP, by which recruits Caspase-9. This recruitment process activates Caspase-9, in turn, downstream Caspases such as Caspase-3 and Caspase-7 (Yu et al., 2005).

Effector Caspases such as Caspase-3, -6 and -7, are activated by the upstream initiator Caspases or other proteases such as granzyme B (Martin et al., 1996) through a proteolytic cleavage. The possibility of autocatalytic activation is not completely discarded though the caspase precursors are present in cells in a conformation or a complex that prevents autocatalysis (Thornberry, 1998). Effector Caspases are activated to complete the execution of apoptosis.

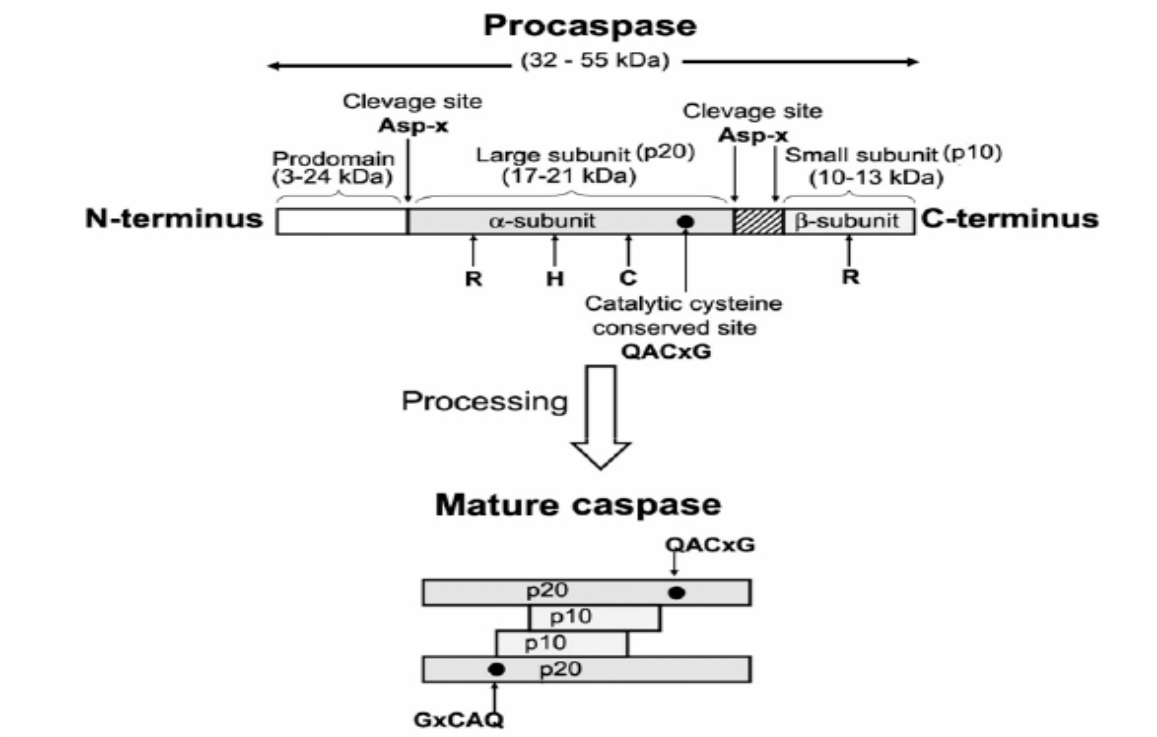


Figure 3. A schematic representation of structural features of mammalian Caspases. C, H and R represent the active site residues (Chowdhury et al., 2008).

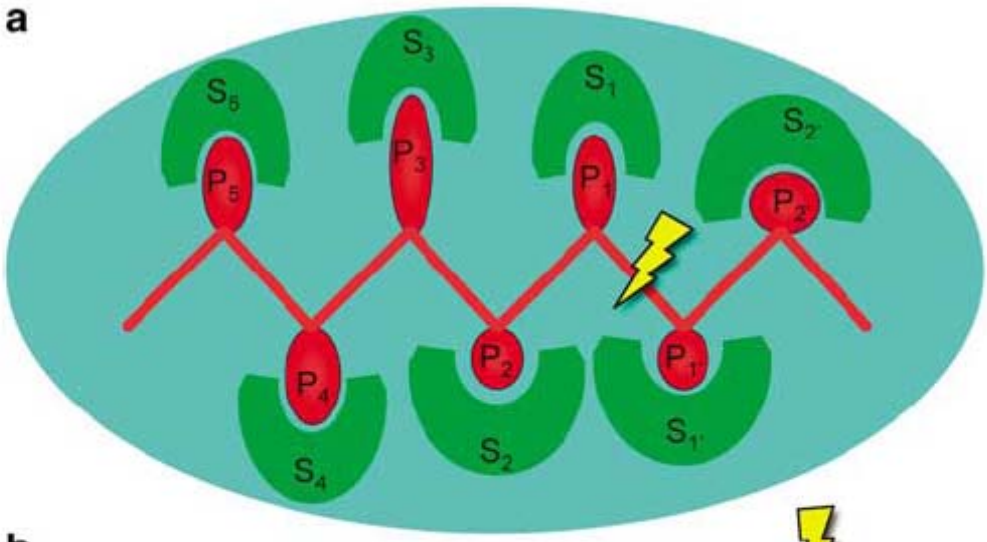
1.2.3 Caspase substrates

Caspases are specific cysteine proteases, recognizing 4 amino acids. The cleavage takes place typically after the C-terminal residue, which is usually an asparagine. And usually from N-terminal the second position is a glutamine for all mammalian Caspases. Therefore the substrate specificity can be described as X-Glu-X-Asp. Caspase-1, -4, and -5 prefer the tetrapeptide sequence WEHD. Caspase-2, -3, and -7 have a preference for the substrate DEXD, whereas Caspase-6, -8, and -9 prefer the sequence (L/V) EXD. It is of interest that the cleavage site between the large and small subunits for initiator Caspases carries its own tetrapeptide recognition motif, which is remarkably consistent with the proposed mechanism of autoactivation of initiator Caspases (Chowdhury et al., 2008; Lavrik et al., 2005b) (Figure 4).

There are many proteolytic events owing to the continual proliferation. Given to the complexity of the animal proteomes it is difficult to identify functionally important Caspase substrates from the proteolytic noise. Some assays involving K_{cat}/K_m calculation of protein cleaved by recombinant Caspases *in vitro*, proteins the mapping of precise Caspase cleavage sites, cleavage site mutant protein creation, knock-out and transgenic generation in cell or animal level are used to generate a definitive link between proteolysis of an individual substrate and a specific 'apoptotic parameter'. To date, almost 400 substrates for the apoptosis-associated Caspases have been reported and there are likely to be hundreds more yet to be discovered. Here we only highlight a few below (Luthi and Martin, 2007; Timmer and Salvesen, 2007).

Executioner procaspase zymogens. Caspase-3, -7 are substrates for the initiator Caspases. Their cleavage sites fit the Caspase-8 and -9 consensus (Thornberry et al., 1997). This cleavage induces their activation.

Bid. Caspase-8 has the same efficiency to cleave Bid as procaspase-3 *in vitro* (Bossy-Wetzel and Green, 1999). There are two putative cleavage sites in Bid, $LQTD_{60}\downarrow G$ and $IQAD_{75}\downarrow S$ (McDonnell et al., 1999). Caspase-8 only cleavage the first possible bond $LQTD_{60}\downarrow G$ rather than $IQAD_{75}\downarrow S$. The $IQAD_{75}\downarrow S$ can be cleaved by Granzyme B. Both of cleavage can promote the apoptosis (Barry et al., 2000).



Caspase	P_5	P_4	P_3	P_2	P_1	P_1'
1, 4, 5, 14		W/Y	E	X	D	Φ
8, 9, 10		I/L	E	X	D	Φ
3, 7		D	E	X	D	Φ
6		V	E	X	D	Φ
2	V/L	D	E	X	D	Φ

Figure 4. Inherent substrate specificity. The convention in naming substrate residues in protease active sites is to name the residue to the N-terminal of the scissile bond (lightning bolt) P1 and the one to the C-terminal side P10. (a) Other residues (red) are numbered consecutively from these origins. Many of these residues will occupy specific pockets on the enzyme, numbered with the matching 'S' designation (green), each of which may be constructed of several enzyme residues. The objective of this binding mode, which almost always binds the cleavage region in an extended peptide conformation, is to align the substrate accurately into register with the catalytic machinery. (b) Caspases bind substrates through typical interactions of the active site cleft with amino acids in the cleavage site. Studies with synthetic peptides and peptide libraries have shown that substrate residues P4 through P10 contribute to binding through association with S4 through S10 pockets in the active site. All Caspases show exquisite selectivity for Asp in the P1 position, a preference for Glu in P3, and a lack of tolerance for charged residues at P10 where Gly, Ala, Thr, Ser and Asn are preferred (denoted by the f symbol). Distinguishing between them is primarily the nature of the P4 residue, and the inherent substrate specificities are generally well understood at the atomic level. Importantly Caspase-2 has an additional interaction at S5 that enhances catalysis at least 30-fold (Timmer and Salvesen, 2007).

eIF4GI. In eukaryotic cells the polypeptide chain initiation factor eIF4G plays an essential role in the mechanism of translation by acting as a molecular bridge between other components of the ribosomal initiation complex. Two forms of eIF4G, eIF4GI and eIF4GII, which are products of different genes, have been identified. Polypeptide chain initiation factor eIF4GI undergoes Caspase-mediated degradation during apoptosis to give characteristic fragments. The most prominent of these has an estimated mass of approximately 76 kDa (Middle-Fragment of Apoptotic cleavage of eIF4G; M-FAG) (Bushell, Poncet et al. 2000). Using a HeLa-based cell-free translation system that mirrors the function of cellular IRES *in vitro*, multiple cellular IRES-dependent translation of Pro- and antiapoptotic proteins (but not cap-dependent) is fully restored by addition of the conserved middle fragment of eIF4GI with the deletion of eIF4GI (Hundsdoerfer, Thoma et al. 2005).

DEF45/ICAD. DEF45/ICAD (DNA fragmentation factor 45/inhibitor of Caspase activated DNase) is the chaperone/inhibitor of DEF40/CAD (DNA fragmentation factor 40/Caspase activated DNase), which is responsible for double-strand cleavages in apoptosis. The complex is disrupted when Caspase-3 cleaves the ICAD at $DETD_{117}$↓S and $DEVD_{224}$↓T. The functional loss of ICAD will allow the nuclease CAD to dimerize into the catalytic form inducing DNA fragmentation (Otomo et al., 2000; Sakahira et al., 1998).

Poly (ADP-ribose) polymerase. Poly (ADP-ribose) polymerase (PARP) is a nuclear protein which involve in the DNA repair. It can catalyze Poly (ADP-ribose) ligation to acceptor protein including itself. During apoptosis, PARP is one of the earliest targets for cleavage by the apoptotic Caspases, resulting in formation of 24 and 89 kDa fragments. Caspase-3 and 7 cleave it at position $DEVD_{213}$↓G (Kaufmann et al., 1993; Lazebnik et al., 1994). Mice expressing a transgenic non cleaved mutant ($DEVN_{213}$↓G) PARP develop normally, but resist to endotoxic shock and intestinal and renal ischemia-reperfusion *in vivo* (Petrilli et al., 2004).

1.2.4 Synthetic Caspase inhibitors

Synthetic Caspase inhibitors have a very important role in research and clinical use. Because Caspases are cysteine proteases, any inhibitors that can react with the catalytic cysteine are also capable of inhibiting Caspases. Until now many synthetic Caspase inhibitors have been developed.

A number of specific and broad-spectrum peptide Caspase inhibitors have been developed, the first based upon Caspase substrates, for example, the YVHD cleavage site in pro- IL1b. These inhibitors act as pseudosubstrates for active Caspases and are therefore competitive inhibitors.

Peptides have been linked to chemical groups that serve to block the amino acids and to improve cell permeability, stability and efficacy. These peptide inhibitors range from a single O-methyl-aspartate residue (e.g. benzyloxycarbonyl- aspartyl (OMe)-fluoromethylketone: Boc-Asp-FMK) to tri-peptides (e.g. Boc-val-ala-asp (OMe)-fluoromethylketone: z-VAD-FMK) and tetrapeptides (e.g. YVAD-FMK).

Peptides linked to aldehydes (or nitriles or ketones) are reversible inhibitors (e.g. Ac-DEVD-CHO, Ac-YVAD-CHO) and bind to the catalytic site, but do not irreversibly chemically alter the enzyme.

Peptides linked to leaving groups (an ion or substituent with the ability to detach itself from a molecule) such as halomethylketones (chloro or fluoro), acylomethyl ketones and (phosphinyloxy) methyl ketones are irreversible inhibitors. It has been proposed that irreversible inhibitors (e.g. Ac-DVAD-FMK) bind via a thioester linkage to a transition state oxyanion and the carbonyl oxygen occupies an oxyanion hole in the Caspase transition state (Callus and Vaux, 2007).

In summary, Synthetic peptide inhibitors that have been developed act by binding to the active site of Caspases either in a reversible or irreversible manner. Inhibitor design includes a peptide recognition sequence attached to a functional group such as an aldehyde (CHO), chloromethylketone (CMK), or fluoromethylketone (FMK). Caspase inhibitors with CMK or FMK group are irreversible while those with CHO group are reversible. In general, protease inhibitors with FMK group are less reactive than those with CMK group and therefore are considered as more specific for the enzyme site being inhibited (Otto and Schirmeister, 1997).

Caspase inhibitor	Source	Target caspase(s)	Strength of inhibition (where known)
Q-VD-Oph	Synthetic peptidic	Caspases 1, 3, 8 & 9	IC50 25–400 nM
DEVD-CHO	Synthetic peptidic	Strong inhibitor of caspase 3 and caspase 7	K_i <2 nM
zVAD-FMK	Synthetic peptidic	Inhibits all caspases, but inhibits caspase 2 very weakly	
IDN-6556	Synthetic non-peptide, Idun (Pfizer)	Broad spectrum irreversible caspase inhibition	IC50 25 nM
Pralnacasan	Synthetic non-peptide (Vertex)	Caspase 1	
M867	Synthetic non-peptide (Merck-Frosst)	Reversible, selective effector caspase inhibitor	K_i 1.4 nM (casp 3) K_i 8.9 nM (casp 7)
Compound 34	Synthetic non-peptide (Sunesis)	Targets allosteric site rather than active site	Inhibits caspase 1 and caspase 7 and possibly others

Table 1. Specificity of chemical Caspase inhibitors (Callus and Vaux, 2007)

The peptide Caspase inhibitors are able to block Caspase activity *in vitro* with Ki's down to the high picomolar ± low nanomolar range (Table 1). The halomethyl ketone-linked peptide YVAD has similar kinetics to the aldehyde linked peptide. Ac-WEHD-CHO, Ac-DEVD-CHO, Ac-YVAD-CHO, t-butoxycarbonyl-IETD-CHO, and t-butoxycarbonyl- AEVD-CHO display a wide range of selectivity and potencies against Caspases, with dissociation constants ranging from 75 pM to 410 mM. The results obtained with peptide-based inhibitors are in accord with those predicted from the substrate specificity studies.

Although these protease inhibitors are more specific than general cysteine protease inhibitors such as iodoacetamide, z-VAD-FMK, z-DEVD-FMK and Ac-YVAD-CMK are all capable of efficiently inhibiting other proteases, such as cathepsin B, both *in vitro* and in tissue culture at concentrations that are generally used to demonstrate the involvement of Caspases. YVAD-CHO is probably the most specific of the commonly used Caspase inhibitors as it only potently inhibits Caspases. The other Caspase inhibitors do not target individual Caspases. For example, while it is not usually regarded as a broad range Caspase inhibitor and most potently inhibits Caspase-3, DEVD-CHO also strongly inhibits Caspase-7 and -8. z-VAD-CHO can strongly inhibit Caspase-1, -3, -5, -7, -8, and -9, but is not a good inhibitor of Caspase-2 (Ekert et al., 1999).

1.3 The apoptotic signaling pathway

Apoptosis is mediated by two central pathways: the extrinsic (or death receptor) pathway and intrinsic (or mitochondrial) pathway.

1.3.1 The extrinsic pathway

The extrinsic pathway of apoptosis is induced by a complex including death ligands and death receptors. The death receptors are a family of type I transmembrane proteins characterized by the presence of multiple cysteine-rich repeats in the extracellular domains and the protein-protein interaction module known as the death domain (DD) in the cytoplasmic tails. The members of death receptor contain tumor necrosis factor receptor 1 (TNFR1; also known as DR1, CD120a, p55 and p60), Fas (also known as DR2, APO-1 and CD95), DR3 (also known as APO-3, LARD, TRAMP and WSL1), TNF-related apoptosis inducing ligand and receptor 1 (TRAILR1; also known as DR4 and APO-2), TRAILR2 (also known as DR5, KILLER and TRICK2), DR6, ectodysplasin A receptor (EDAR) and nerve growth factor receptor (Lavrik et al., 2005a). Different death receptors are triggered by corresponding ligands, by which the death domain recruits a number of molecules activating the signaling cascade. Two activating models have been described (Figure 5).

1.3.1.1 CD95 and TRAILR pathway

Upon interacting with their respective ligands, CD95 and TRAILR1/TRAILR2 undergo multimerization to from the intracellular death-inducing signaling complexes (DISCs). FADD (Fas Associated Death Domain), a adaptor protein, contain DD (death domain) and DED (death effector domain) (Peter and Krammer, 2003). Death domain of FADD interacts with death domain of death receptors; on the other hand the DED of FADD recruits the procaspase-8 via interaction with the N-terminal tandem DED of procaspase-8. The recruiting of procaspase-8 leads to a high local concentration which results in the autoproteolytic activation. Activated Caspase-8 is released to the cytosol to propagate the apoptosis.

Procaspase-10 is also activated at DISC (Sprick et al., 2002), but it is still controversial whether Caspase-10 can induce the cell death in the absence of Caspase-8.

$FLIP_{L/S}$ can inhibit the Caspase-8 activation by the competitive interaction with DED of FADD. However, some labs reported that FLIPL also can facilitate the

cleavage of the procaspase-8 at the DISC by forming FLIPL-procaspase-8 heterodimers.

In addition to the molecules mentioned above, some other proteins are also recruited by the DISC including Daxx, FAP-1, FLASH, RIP, FAF-1 etc. (Peter and Krammer, 2003).

Type I and II CD95 apoptotic signaling have been established. In type I cells activated Caspase-8 directly results in the activation of downstream executioner Caspases due to the high level of DISC. But for the type II cells there are less DISC formation and activated Caspase-8. In this case Caspase-8 cleaves the Bid to truncated Bid (tBid), which involve in the release of cytochrome c (Cyt c) from mitochondria to form the apoptosome with Apaf-1 and procaspase-9 in cytosol. Caspase-9 activates the downstream effector Caspases (Korsmeyer et al., 2000; Scaffidi et al., 1998). TRAILR1/R2 signaling is similar with type II CD95 signaling. Some anti-apoptotic proteins can block the type II signaling such as Bcl-2 and Bcl-XL (Willis et al., 2003).

1.3.1.2 TNFR pathway

TNF receptors comprise two isoforms named TNF-R1 (also known as p55TNFR, p60, CD120a, TNFRSF1a) and TNF-R2 (also known as p75TNFR, p80, CD120b, TNFRSF1b), which share only the 28% of homology mostly in their extracellular portion. TNFR-1 contains Death Domain (DD) in the intracellular part whereas this DD part is absent in TNFR-2 (MacEwan, 2002; Yang et al., 2002). TNFR-1 (p60) is expressed on all cell types, while TNFR-2 (p80) expression is mainly confined to immune cells (Aggarwal, 2003). For this reason, TNFR-1 is an important member of the death receptor family that shares the capability of inducing apoptotic cell death (Ashkenazi and Dixit, 1998).

TNF-R1 is stimulated by TNFα and recruits the adaptor protein named TNFR–Associated Death Domain (TRADD), by which form a complex I by recruiting RIP (receptor-interacting protein), TRAF-1/2 (TNFR associated factor) and probably other unidentified molecules. Complex I is supposed to trigger the signaling pathway via recruitment of the IKK complex and activates JNK through a TRAF-2-dependent mechanism. Complex I without TNF and TNF-R1 can translocate to the cytosol, where FADD, procaspase-8/10 and $FLIP_{L/S}$ are recruited to form the so-called

complex II or traddosome inducing Caspase-8 activation. Activated Caspases-8 promotes the apoptotic signaling by activating the downstream effector Caspases (Micheau and Tschopp, 2003).

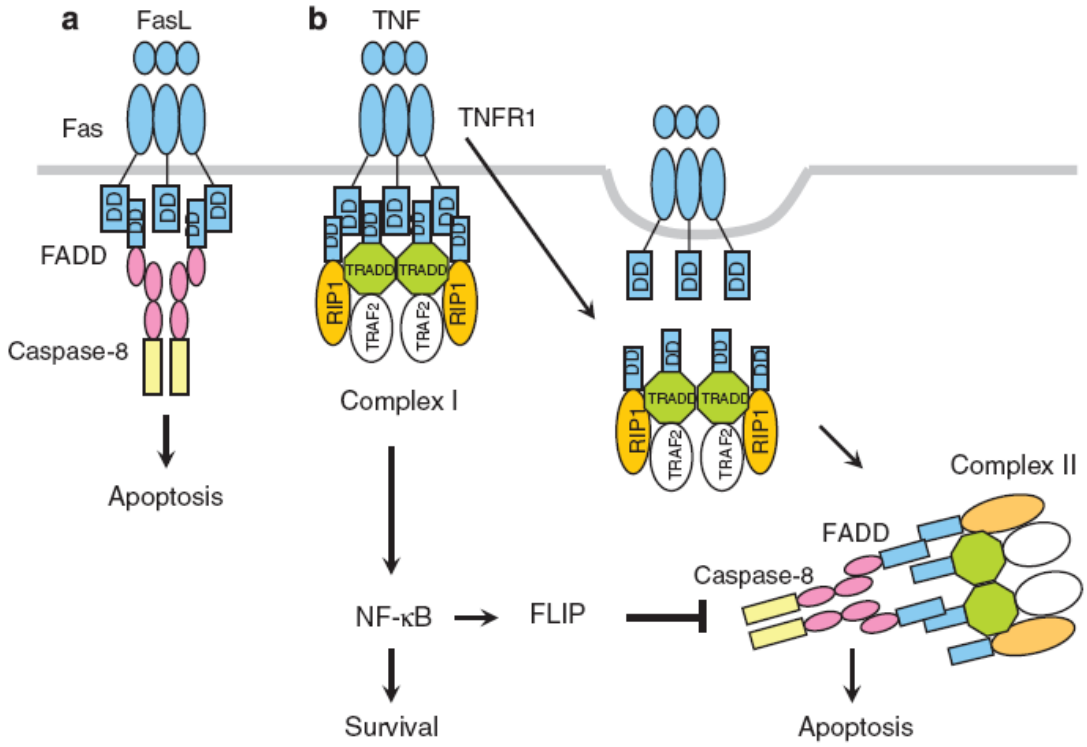


Figure 5. Death-inducing signaling complex (DISC). (a) DISC of Fas and TRAIL receptors. Death domain (DD) of Fas recruits the adapter protein FADD. FADD in turn, via its death effector domain (DED), recruits and activates Caspase-8. (b) DISC of tumor necrosis factor (TNF) receptor 1. After the binding of TNF to TNFR1, rapid recruitment of TRADD, RIP1 and TRAF2 occurs (complex I). Subsequently, TNFR1, TRADD and RIP1 are modified and dissociate from TNFR1. The liberated DD of TRADD (and/or RIP1) binds to FADD, resulting in Caspase-8 recruitment (complex II) and apoptosis. Complex I activates nuclear factor-κB (NF-κB) and promotes FLIP expression, which inhibits Caspase-8 and antagonizes apoptosis (Li and Yuan, 2008).

Due to TNFR-2 lacking a cytoplasmic DD comparing with TNFR-1, interaction between TNFα and TNFR-2 results in binding of TRAF-1/2 to the cytoplasmic portion of TNFR-2. Then, this recruits the cellular inhibitor of apoptosis proteins

cIAP-1 and cIAP-2 (Deveraux et al., 1999), which activates NF-κB signaling pathway to inhibit apoptosis and provide a survival signal. However, it has been reported that TNFR-2 may also play an important role in the regulation of apoptosis through TNFR-1 (Gupta and Gollapudi, 2005).

1.3.2 Intrinsic pathway

In contrast to the extrinsic pathway that is induced by extracellular stimuli, the intrinsic pathway integrates a broad spectrum of extracellular and intracellular stresses. The intracellular stimuli include DNA damage, oxidative stress and protein misfolding and extracellular stimuli include the starvation, radiation and cytotoxic drugs.

1.3.2.1 Mitochondria

Mitochondria have various critical cellular functions including generation of ATP through the process of aerobic respiration. However, other mitochondrial functions such as the generation of acetyl CoA, nicotinamide adenine dinucleotide production and buffering intracellular calcium levels are also important. In the intrinsic apoptosis pathway mitochondria is a central organelle. Its dysfunction and release of mitochondrial proteins to the cytosol initiate the apoptotic cascade.

1.3.2.2 The apoptosome as activating complex for procaspase-9

Upon stress pro-apoptotic proteins overcome inhibitory effects of anti-apoptotic proteins located in the mitochondria membrane inducing the release of cytochrome c from mitochondria to cytosol (Chipuk and Green, 2008). With the appearance of cytochrome c in the cytosol, Apaf-1, an adaptor protein, assembles with procaspase-9, cytochrome c and the cofactor dATP/ATP (Liu, Kim et al. 1996; Li, Nijhawan et al. 1997; Zou, Henzel et al. 1997). Apaf-1, a 140kDa central scaffold protein of the apoptosome, contains the N-terminal CARD domain which interact with CARD of procaspase-9 and a C-terminal WD40 domain which interacts with cytochrome c and dATP. In the presence of cytochrome c and dATP, Apaf-1 oligomerizes to form a very large (700-1400kDa) wheel-shaped apoptosome complex. Procasapse-9 dimerizes to be activated, in turn, activates the downstream effector Caspases such as

Caspase-3 and Caspase-7 (Acehan, Jiang et al. 2002; Boatright, Renatus et al. 2003) (Figure 6).

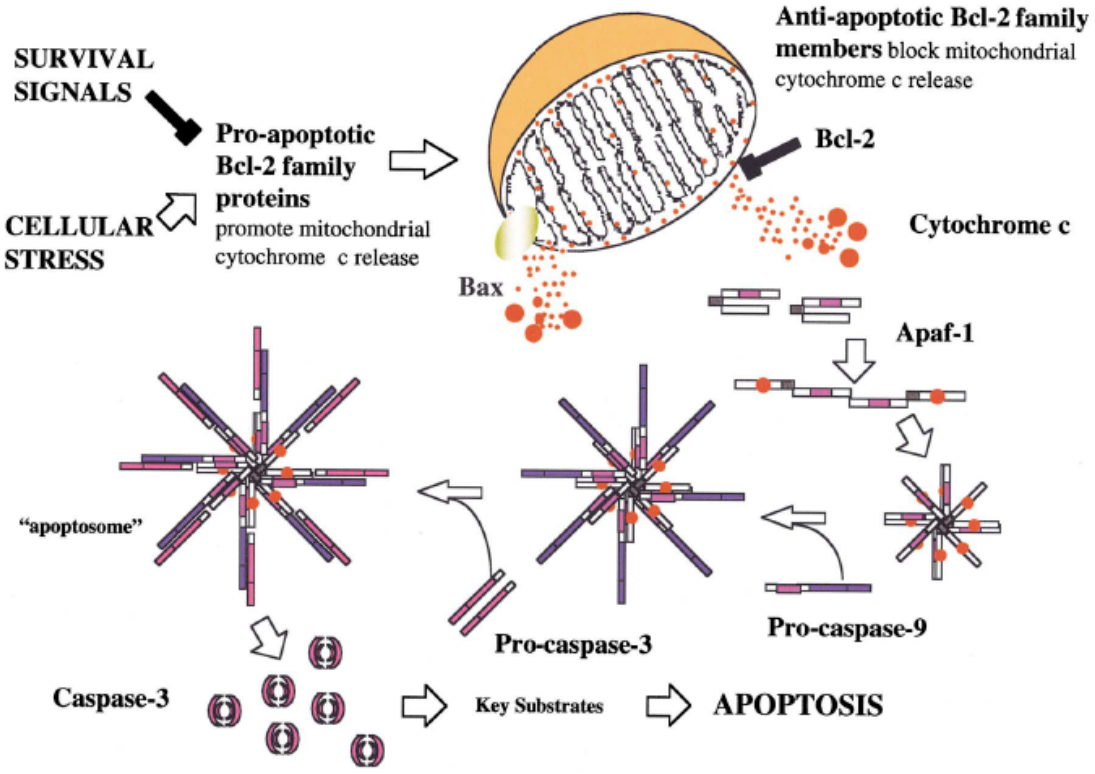


Figure 6. Induction of apoptosis via the mitochondrial pathway. Cellular stress induces pro-apoptotic Bcl-2 family members to translocate from the cytosol to the mitochondria, where they induce the release of cytochrome c. Cytochrome c catalyzes the oligomerization of Apaf-1, which recruits and promotes the activation of procaspase-9. This, in turn, activates procaspase-3, leading to apoptosis (Zimmermann et al., 2001).

1.3.3 Mitochondrial outer membrane permeabilization (MOMP) and mitochondrial permeability transition pore (MPTP)

The release of mitochondrial proteins is a key step inducing the intrinsic pathway of apoptosis. Its release depends on the MPTP and MOMP, which are regulated by the Bcl-2 family of proteins.

The activation of Caspases in the mitochondrial pathway can be induced by mitochondrial outer membrane permeabilization (MOMP), an event that is considered to be the 'point of no return' during apoptosis as it results in the diffusion to the cytosol of numerous proteins that normally reside in the space between the outer (OMM) and inner (IMM) mitochondrial membranes (Von Ahsen et al., 2000). Among these are cytochrome c, AIF and EndoG etc., by which induce formation of apoptosome with Apaf-1 and procaspase-9 inducing apoptosis or inducing Caspase-independent cell death (Susin, Lorenzo et al. 1999; Li, Luo et al. 2001; Stefanis 2005). The MOMP is described as a Bax/Bak-lipid pore. Activation by certain BH3-only proteins is required for Bax or Bak to oligomerize and insert stably into the OMM (Kuwana, Mackey et al. 2002) (Figure 7). The Bax/Bak double knockout cells are resistant to many proapoptotic stimuli that act through disruption of mitochondrial function: staurosporine, ultraviolet radiation, growth factor deprivation, etoposide, and the endoplasmic reticulum stress stimuli thapsigargin and tunicamycin (Wei, Zong et al. 2001).

Several genetic models have shown the function of these components of MPTP. ANT seems to be dispensable as mice lacking ANT can still undergo MPTP (Kokoszka, Waymire et al. 2004). VDACs are also dispensable for both MPT and Bcl-2 family member-driven cell death. *Vdac1*-, *Vdac3*-, and *Vdac1–Vdac3*-null mice show no differences with the wild type (Baines, Kaiser et al. 2007). But the same lab found that cyclophilin D plays a critical role for mitochondrial permeability transition in cell death. Ppif null mice (the gene encoding cyclophilin D) are protected from ischemia/ reperfusion-induced cell death *in vivo*, whereas cyclophilin D overexpressing mice show mitochondrial swelling and spontaneous cell death. Mitochondria isolated from the livers, hearts and brains of Ppif null mice are resistant to mitochondrial swelling and permeability transition *in vitro*. Moreover, primary hepatocytes and fibroblasts isolated from Ppif null mice are largely

protected from Ca^{2+}-overload and oxidative stress-induced cell death (Baines, Kaiser et al. 2005).

The MPTP (mitochondrial permeability transition pore) is a complex composed of several different proteins including VDAC (voltage-dependent anion channel), ANT and cyclophilin D (cypD), which span the IMMs and OMMs where ANT is on the inner membrane and VDAC is on the outer membrane (Figure 8). Opening of this

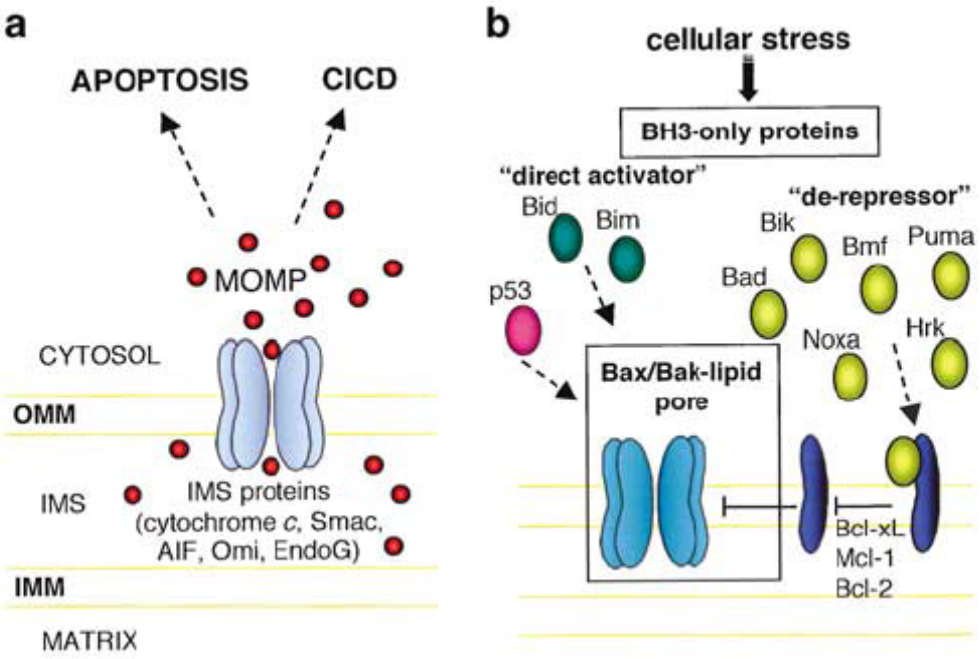


Figure 7. (a) Schematic representation of MOMP. Diverse forms of cellular stress activate the mitochondrial pathway by inducing the formation of pores in the OMM. Permeabilization of the OMM allows for the release of numerous intramembrane space proteins, which results in the activation of Caspases and apoptosis; or it may induce death in a Caspase-independent manner (CICD). (b) The BAX/BAK-lipid pore model. Direct activator BH3-only proteins or p53 induce BAX or BAK to form pores in the OMM. BCL-2, BCL-xL and MCL-1 can block this process at the OMM and are, in turn, regulated by de-repressor BH3-only proteins (Chipuk et al., 2006)

pore allows for an influx of ions and other small molecules into the mitochondrial matrix causing swelling of the matrix, inducing rupture of the OMM and thus MOMP. MPTP opening can be triggered under stress conditions, however, by increases in Ca^{2+}, oxidative stress, depletion of adenine nucleotides, increases in inorganic phosphate, and depolarization of the inner mitochondrial membrane,

stimuli that operate during ischemia-reperfusion (Halestrap et al., 2002). Cytochrome c release caused by MPT-mediated rupture of the outer mitochondrial membrane may occur during necrosis and forms of apoptosis associated with mitochondrial calcium overload including ischemia-reperfusion (Kim et al., 2003).

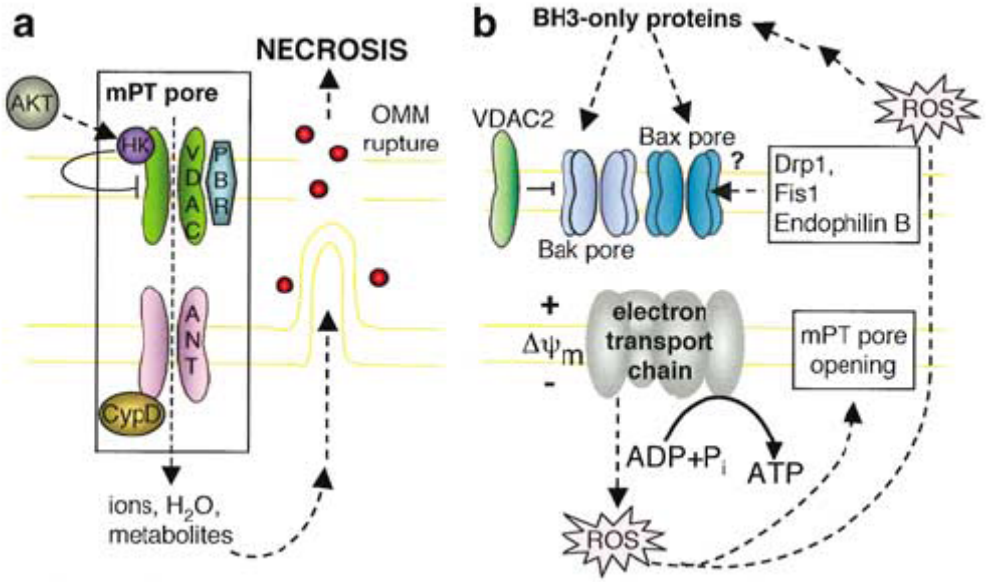


Figure 8. (a) Schematic representation of the mPT pore model. The hypothetical mPT pore is comprised of VDAC, ANT and a number of other proteins. Opening of the pore allows for an influx of water and ions into the matrix inducing swelling and rupture of the OMM and most likely results in necrosis. Various proteins have been suggested to regulate the mPT including hexokinase (HK) and the PBR. (b) Other proteins have been suggested to regulate MOMP, specifically the formation of the BAX (or BAK) pore including VDAC2 and proteins that regulate mitochondrial fission and fusion. Further, mitochondrial components may independently induce MOMP including ROS produced by the electron transport chain, which may cause opening the mPT pore, but equally may induce apoptosis via activation of BH3-only proteins in the cytoplasm (Chipuk et al., 2006)

Three models of mitochondrial apoptogen release have been proposed (Figure 9) (Crow, Mani et al. 2004). But it is not very clear yet.

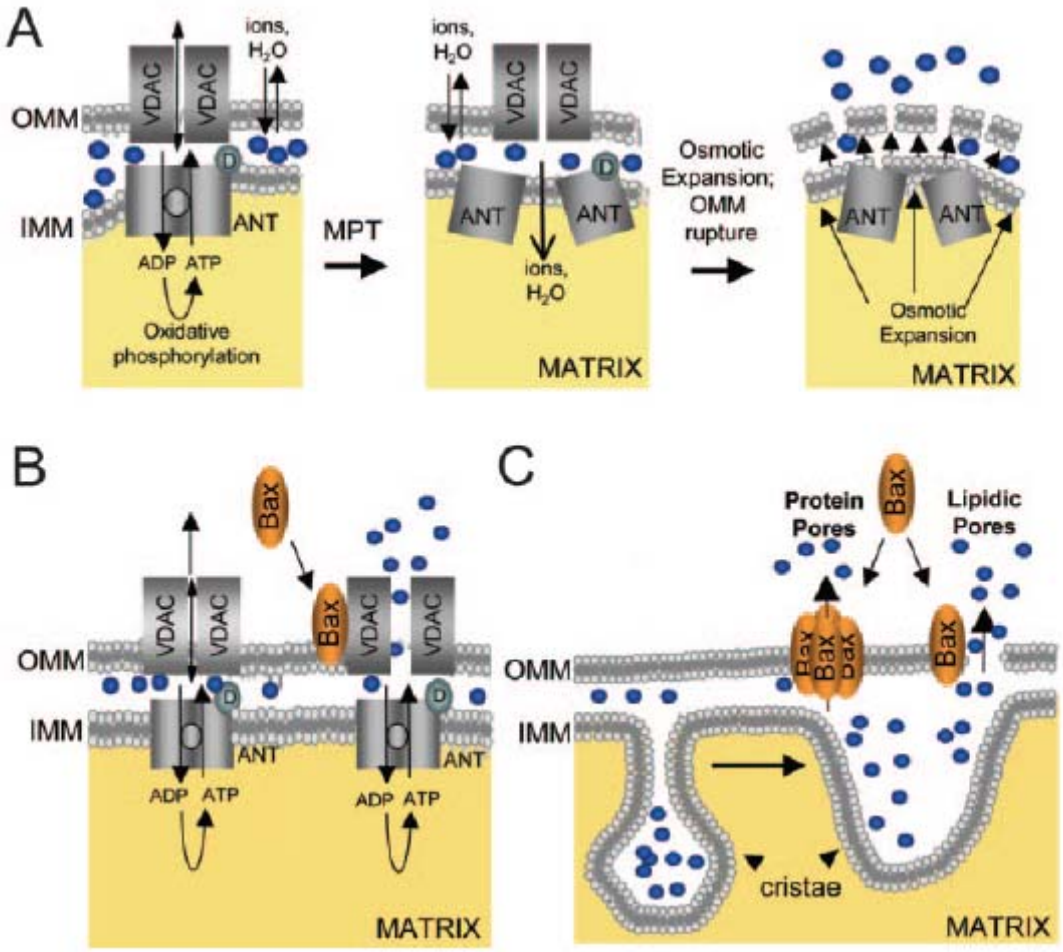


Figure 9. Models of cytochrome c release. A, MPT model. In healthy cells (left panel), the MPTP functions as a nucleotide exchanger. In response to stress signals, ANT undergoes a conformational change that converts it from a ligand-specific translocase to a nonspecific pore that collapses the inner mitochondrial membrane (IMM) potential and allows the entry of water down its osmotic gradient (middle panel). The subsequent swelling of the IMM ruptures the outer mitochondrial membrane (OMM), releasing cytochrome c (blue) (right panel). D denotes cyclophilin D. B, Bax–VDAC model. In healthy cells (left), VDAC functions as a component of the nucleotide exchange complex and restricts the passage of cytochrome c. During apoptosis (right), Bax binds VDAC changing its properties to permit cytochrome c release. C, Bax pore models. In healthy cells (left), ~15% of cytochrome c is in the mitochondrial intermembrane space and ~85% in cristae that are sequestered from the intermembrane space because of narrow junctions. During apoptosis, remodeling of cristae widens the junctions, allowing the majority of cytochrome c to access the intermembrane space. Bax is postulated to form protein pores or to stimulate the formation of lipid pores in the OMM, through which cytochrome c translocates into the cytosol (Crow, Mani et al. 2004).

1.3.4 Bcl-2 family members

The Bcl-2 family of proteins is a group of evolutionarily conserved regulators of cell death, comprising both anti- and pro-apoptotic members, which regulate the apoptosis. Members share up to four conserved regions of homology, or Bcl-2 homology domains (BH1, BH2, BH3 and BH4), which mediate interactions between the family members (Table 2). The Bcl-2 family can be divided into the following: (1) antiapoptotic members (eg, Bcl-2 and Bcl-XL [Bcl-x protein long isoform]) and (2) proapoptotic members, which are further subdivided into (a) multidomain proapoptotic (eg, Bax [Bcl-2–associated X protein] and Bak [Bcl-2–antagonist/killer]) and (b) BH3-only proapoptotic (eg, Bid, Bad [Bcl-2-antagonist of cell death], Bim [Bcl-2 interacting mediator of cell death], Bmf [Bcl-2 modifying factor], Noxa [for damage], Puma [p53 upregulated modulator of apoptosis], BNip3 [Bcl-2/adenovirus E1B nineteen kDa-interacting protein 3], and Nix [Nip3-like protein X]/BNip3L [BNip3-like protein]). The antiapoptotic and multidomain proapoptotic contain BH1–3 domains. In addition, some, but not all, of the antiapoptotic contain a BH4 domain. The BH3-only proteins are homologous to these two groups only in the 10 to 16 residue BH3 domain (Crow, Mani et al. 2004).

Classification	Domains	Members
Anti-apoptotic	BH4 BH3 BH1 BH2 TM Contain 2 or 4 BH domains and a TM domain	Bcl-2, Bcl-x_L, Bcl-w, Mcl-1, A1
Pro-apoptotic	BH3 BH2 BH1 TM Contain 2 or 3 BH domains and a TM domain	Bax, Bak, Bok, Bcl-x_s
BH3-only	BH3 TM BH3 domain and a TM domain	Bim, Bik, BNIP3, BNIP3L,
	BH3 BH3 domain	Bid, Bad, Noxa, Puma, BMF, Beclin-1

Table 2. Bcl-2 Family (Mellor and Harris, 2007)

1.3.4.1 Antiapoptotic Bcl-2 proteins

Antiapoptotic Bcl-2 family members including Bcl-2 and Bcl-XL can antagonize the multidomain and BH3-only proapoptotic Bcl-2 proteins. Bcl-2 has been found residing in the outer mitochondrial membrane, endoplasmic reticulum (ER), and nuclear envelope (Krajewski, Tanaka et al. 1993; Lithgow, van Driel et al. 1994) facing the cytoplasma. Bcl-XL is also found at these membranes, but the majority is in the cytosol (Hsu and Youle, 1997). Antiapoptotic Bcl-2 family proteins probably antagonize proapoptotic proteins by direct interaction with Bax or Bak. But some data show that Bcl-2 and Bax can regulate cell death independently (Knudson and Korsmeyer, 1997). Bcl-XL mutant without Bax and Bak binding domain is still protective whereas Bcl-XL mutant lacking of tBid binding domain is capable of protection (Cheng, Wei et al. 2001). It indicates the indirect way involving in the BH3-only proteins.

Bcl-2 and Bcl-XL appear to control cell survival beyond the Apaf-1-Caspase-9 axis. The Apaf-1 or Caspase-9 or double deficiency can delay but eventually induce cell death. In contrast, transfection with Bcl-2 provided long-term, clonogenic protection, and could act independently of the apoptosome (Ekert, Read et al. 2004; Marsden, Kaufmann et al. 2006). The disruption of mitochondria may be the final cause determining the cell death. The ratio of Bcl-2 or Bcl-XL to Bax or Bak plays a key role between the cell survival and death in many cell models.

Antiapoptotic Bcl-2 proteins have shown the important role in development. Bcl-x-/- mice (in which the entire Bcl-x locus (incorporating Bcl-XL and Bcl-XS) was knocked out) survive only until fetal day 13.5, displaying severe defects in erythropoiesis and neuronal development (Motoyama, Wang et al. 1995). By contrast, although Bcl-2-deficient mice survive to birth, they have defects in the immune system, hair follicles and renal epithelial cells, and all succumb to polycystic kidney disease by ~4–8 weeks of age (the age of death is influenced by genetic background) (Veis, Sorenson et al. 1993; Bouillet, Cory et al. 2001). Antiapoptotic Bcl-2 family proteins also can affect myocardial ischemia reperfusion injury. Transgenic mice with cardiac-restricted overexpression of Bcl-2 demonstrated 48% to 64% decrease in infract size (Brocheriou, Hagege et al. 2000; Chen, Chua et al. 2001).

1.3.4.2 Multidomain Proapoptotic Bcl-2 proteins

Either Bax or Bak is required for all instances of apoptosis mediated via the intrinsic pathway (Wei, Zong et al. 2001). These proteins undergo marked conformational changes upon apoptotic stimuli. Bax is mostly cytosolic and sequesters its hydrophobic C-terminal membrane anchor in its BH3-binding pocket, with a minor fraction lightly bound to the OMM (Hsu et al., 1997). On apoptotic stimuli the C-terminal exposure triggers Bax translocation to mitochondria, oligomerization and insertion into the OMM. Bax then induces the release of cytochrome c and other apoptotic protein in the intermembrane of mitochondria (Wolter, Hsu et al. 1997; Goping, Gross et al. 1998). In the case of Bak, it already resides on the OMM, where it has been reported to be bound to OMM channel protein VDAC2 (Cheng, Sheiko et al. 2003), MCL-1 and Bcl-XL (Willis et al., 2005). On the initiation of apoptosis, VDAC2 is displaced from Bak by activated Bid, Bim, and Bad, whereas MCL-1 is degraded (Cuconati, Mukherjee et al. 2003; Nijhawan, Fang et al. 2003) and/or Bak-MCL-1 and Bak-Bcl-XL interaction are disrupted by BH3-only proteins, such as NOXA, Bim (Willis et al., 2005) or BIK (Shimazu, Degenhardt et al. 2007), which frees Bak and stimulates its homoligomerization inducing release of apoptogenic mitochondrial proteins into cytosol.

Bax knockout mice are viable and the males have a severe defect in sperm cell differentiation, which results in sterility (Knudson, Tung et al. 1995). Bak knockout mice have no obvious defects detected so far. However, Bax/Bak double knockout mice display various severe defects. A large fraction of Bax/Bak double knockout mice die during embryogenesis or perinatally. The neonates display various developmental deficits such as abnormally increased numbers of lymphoid and myeloid cells which normally die by apoptosis. In addition, Bax/Bak-/- cells are resistant to most of the apoptotic stimuli (Lindsten, Ross et al. 2000; Youle and Strasser 2008).

1.3.4.3 BH3-only Bcl-2 Proteins

BH3 only proteins only contain one BH3 domain which is important in mediating heterodimerization with pro-survival proteins and inducing apoptotic cell

death. BH3 domain is underscored by the identification of many BH3-only containing proteins: Bik, Blk, Hrk, BimL, Bad, Bid, and the C.elegans, EGL-1 except to BNip3 (Ray, Chen et al. 2000).

BH3-only proteins can transmit the upstream apoptotic signals directly or indirectly to Bax or Bak. A subset of BH3-only proteins such as Bid and Bim can directly interact with a Bax and Bak to induce their activation (Wei, Lindsten et al. 2000; Letai, Bassik et al. 2002; Kuwana, Bouchier-Hayes et al. 2005). They are 'direct activators'. For the other BH3-only proteins (eg. Bad, Bik, Bmf, BNip3, Noxa and PUMA) function as a 'De-repressor/sensitizer'. These proteins do not directly interact with Bax or Bak. They can displace the direct activators from the antiapoptotic Bcl-2 proteins, by which induce the release of direct activator and

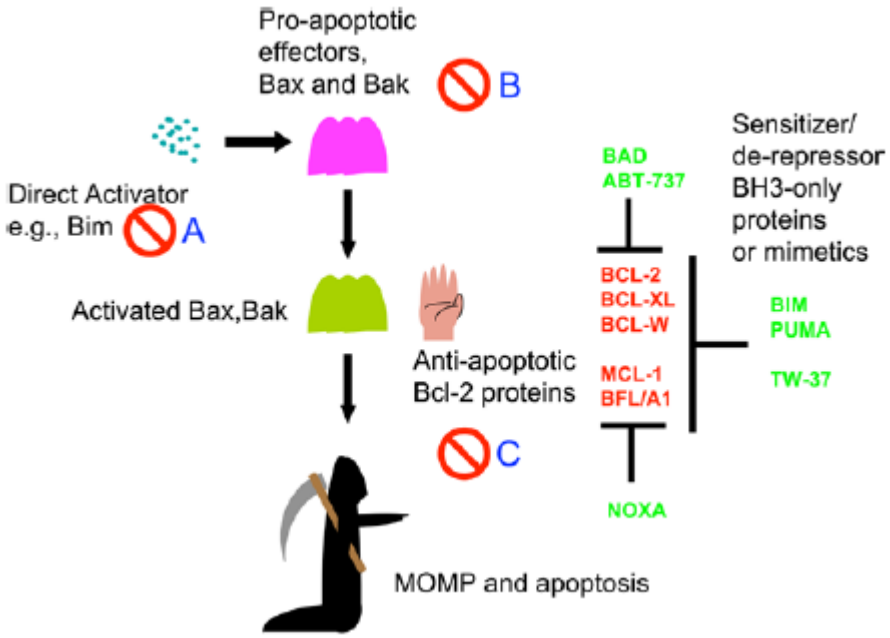


Figure 10. BH3 Profiling. Different BH3-only proteins or their corresponding BH3 peptides effectively neutralize different antiapoptotic Bcl-2 family members, and this may or may not lead to MOMP, depending on the status of the proapoptotic effectors Bax and Bak (the pink "salmon mousse"). These effectors do not promote death on their own, but do so if exposed to direct activator proteins or conditions (the green ptomaine), which include the BH3-only protein BIM but also other proteins as well. Activated Bax and Bak do not trigger MOMP; however, antiapoptotic proteins block MOMP (the hostess preventing the guests from eating), unless these inhibitors are effectively neutralized. Such neutralization can occur by binding to BH3-only proteins or BH3-mimetic drugs. MOMP, when it occurs, leads to cell death (the Grim Reaper). The pattern of MOMP occurrence in response to different BH3-only proteins predicts whether or not the drug ABT-737 will cause apoptosis and defines three classes of resistance to this drug, A, B, and C (Green, 2007).

promote the apoptosis. For example Bim can be released from MCL-1 by Noxa (Czabotar, Lee et al. 2007) (Figure 10).

BNip3 and Nix predominantly localize to mitochondria, which depends on its transmembrane domain (TM) domain (Figure 11). The C-terminal TM domain is not only required for mitochondria location but also for the proapoptotic activity (Boyd, Malstrom et al. 1994; Chen, Ray et al. 1997). The BH3 domain could substitute for Bax , maintaining proapoptotic activity and Bcl-XL heterodimerization (Yasuda et al., 1998). In contrast, Ray et al. found that the BH3 deletion has no effect on the interaction with Bcl-2 and Bcl-XL (Ray, Chen et al. 2000). Interestingly Nix (BNip3-Like protein), another BH3-only protein, shares the ~65% sequence identity to BNip3 (Yasuda et al., 1998) and several key features, including an ability to interact with Bcl-2 and Bcl-XL, inducing apoptosis via its TM domain (Chen et al., 1999).

It has been shown that BNip3 expression is upregulated by the hypoxia in tumor cell line (Sowter, Ratcliffe et al. 2001), and by combination of hypoxia and acidosis in cardiac myocytes and in failing hearts (Guo, Searfoss et al. 2001; Crowley-Weber, Payne et al. 2002; Kubasiak, Hernandez et al. 2002; Regula, Ens et al. 2002). Regulation of Nix by hypoxia in tumor cell line is also reported (Sowter, Ratcliffe et al. 2001; Fei, Wang et al. 2004). The HIF-1 is responsible for the transcriptional upregulation of BNip3 and Nix inducing by hypoxia.

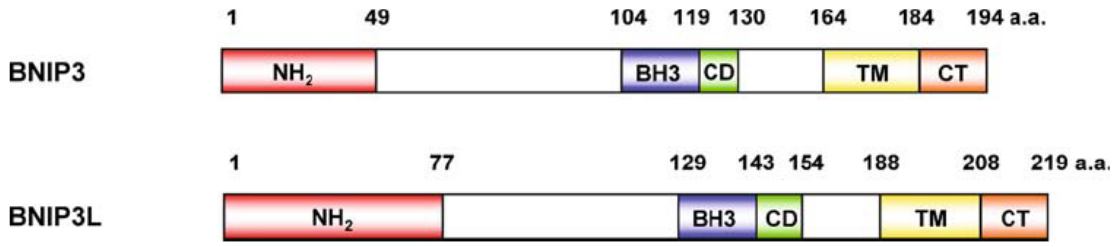


Figure 11. Schematic representation of the domains of BNip3 and BNip3L. Abbreviations: NH2 N-terminal domain, BH3 Bcl-2 homology domain 3, CD conserved domain, TM transmembrane domain, CT COOH-terminal domain (Mellor and Harris, 2007)

The mechanism of BNip3 and Nix induced cell death is not clarified well yet. It probably depends on the cell type and experimental conditions (Chen, Ray et al. 1997; Vande Velde, Cizeau et al. 2000) (Figure 12). One model, which fits most data is that upon activation BNip3 inserts into the mitochondrial outer membrane, and causes opening of the MPTP, loss of $\Delta\Psi_m$, generation of reactive oxygen species, and necrosis. A second possibility is that BNip3 and Nix function as typical BH3-only proteins inducing cell death. A recent study provides evidence for a third potential mechanism of BNip3- or Nix-induced cell death. Bax or Bak, and Bcl-2, have opposing effects on cell death, mediated at least in part through their effects on endoplasmic reticulum/sarcoplasmic reticulum (ER/SR) calcium stores, mitochondrial calcium uptake, and the MPTP (Zhang and Ney, 2009).

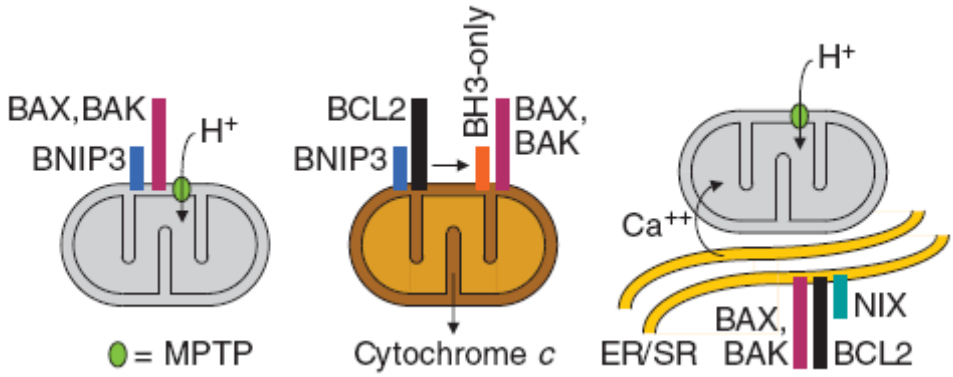


Figure 12. Potential mechanisms of BNip3- and NIX-induced cell death. (Left panel) BNip3 (blue box) or NIX (teal box), in concert with BAX or BAK (plum box), cause opening of the MPTP (lime oval), mitochondrial depolarization (represented as gray color), increased generation of reactive oxygen species, and cell death. (Center panel) BNip3 or NIX bind BCL2 or BCL-XL (black box), releasing BH3-only protein (orange box) to interact with BAX or BAK, causing cytochrome c release, Caspase activation, and cell death. (Right panel) NIX binds to endoplasmic reticulum/sarcoplasmic reticulum (ER/SR), increasing ER/SR calcium (Catt) stores, causing mitochondrial calcium uptake, opening of the MPTP, and cell death (Zhang and Ney, 2009)

In cardiomyocytes, BNip3 induces apoptotic cell death with can be blocked by mitochondrial permeability transition pore (MPTP) inhibitor. The TM of BNip3 plays an important role in inducing cell death and the BNip3 lacking the TM functions as a dominant negative regulator (Kubasiak, Hernandez et al. 2002; Regula, Ens et al. 2002). But contrary to Regula et al., kubasiak et al. reported that the cell

death can not be blocked by Caspase inhibitor. This Caspase-independent cell death is consistent with other reports in 293T cells (Vande Velde, Cizeau et al. 2000) and primary neuron cultures exposed to hypoxia conditions (Zhang et al., 2007b). BNip3-/- mice have shown that BNip3 can minimize ventricular remodeling 3 weeks after I/R (ischemia/reperfusion) and apoptosis was diminished in BNip3-/- periinfarct and remote myocardium 2 days after I/R. Forced cardiac expression of BNip3 increased cardiomyocyte apoptosis in unstressed mice, causing progressive LV dilation and diminished systolic function. Conditional BNip3 overexpression prior to coronary ligation increased apoptosis and infarct size (Diwan, Krenz et al. 2007).

BNip3L-/- mice are viable and fertile, but display profound reticulocytosis and thrombocytosis, massive spenolmegaly and both splenic and bone marrow erythroblastosis. There was no effect of haematopoietic progenitors, but erythroid cells from the mice were hypersensitive to erythropoietin (Epo) and showed reduced apoptosis during erythrocyte maturation suggesting that BNip3L-mediated cell death is important in regulating erythrocyte maturation and haematopoietic homeostasis (Diwan, Koesters et al. 2007). More recently the same lab showed that Nix-mediated cell death involving direct and ER/sarcoplasmic reticulum–mediated (ER/SR-mediated) mitochondria disruption. In genetic mouse models, cardiomyocyte ER/SR calcium stores are proportional to the level of expressed Nix. Whereas Nix ablation was protective in a mouse model of apoptotic cardiomyopathy, genetic correction of the decreased SR calcium content of Nix-null mice restored sensitivity to cell death and reestablished cardiomyopathy. Nix mutants specific to ER/SR or mitochondria activated Caspases and were equally lethal, but only ER/SR-Nix caused loss of the mitochondrial membrane potential.

1.3.5 Proteins released from mitochondria

Mitochondria play a very important role in transmitting and amplifying death signaling to the intrinsic even extrinsic pathway apoptosis. The most critical mitochondrial events during apoptosis are the structural and functional remodeling of this organelle and subsequent release of apoptogenic proteins into cytosol. These proteins include cytochrome c, Smac/DIABLO (second mitochondria-derived activator of Caspase/direct IAP-binding protein with low pI), Omi/HtrA2 (high

temperature requirement protein A2), AIF, and EndoG (Endonuclease G) (Crow, Mani et al. 2004).

Omi/HtrA2

The HtrA family refers to a group of related oligomeric serine proteases that combine a trypsin-like protease domain with at least one PDZ interaction domain. Mammals encode four HtrA proteases, named HtrA1–4. Omi/HtrA2 resides in the mitochondria intermembrane space, and is expressed as a 49kDa proenzyme. A mature form with the cleavage of first 133 aminos exposes the IAP-binding motif (IBM) (Hegde, Srinivasula et al. 2002; Martins, Iaccarino et al. 2002). Mnd2 mice reduced catalytic activity due to a naturally Ser276Cys mutation of Omi/HtrA2 and Omi/HtrA2-/- mice display a striking Parkingsonian phenotype (Jones, Datta et al. 2003; Martins, Morrison et al. 2004). In healthy cells Omi/HtrA2 functionally maintains mitochondria homeostasis. Upon apoptotic stimuli, the release of HtrA2/Omi from mitochondria to cytosol promotes cell death both through IAP inhibition and a Caspase-independent mechanism based on its serine-protease activity (Vande Walle et al., 2008).

Smac/DIABLO

Smac/DIABLO (second mitochondria-derived activator of Caspase/direct IAP-binding protein with low pI) is a 25kDa protein residing in intermembrane of mitochondria with a N-terminal 55 amino acid mitochondria targeting sequence. The sequence is proteolytically cleaved within mitochondria leading to the mature Smac release to cytosol. With release to cytosol Smac promotes the apoptosis via its ability to eliminate IAP-mediated Caspase inhibition (Verhagen, Ekert et al. 2000). Total ablation of Smac/DIABLO of mice has no overt phenotype originally (Okada, Suh et al. 2002). However Smac-deficient oocytes are more prone to die upon cytochrome c injection (Perez, Acton et al. 2007).

AIF

Apoptosis-inducing factor (AIF) is a mitochondrion-localized flavoprotein with NADH oxidase activity that is encoded by a nuclear gene. AIF has been shown to release from mitochondria to cytosol as well as nucleus and induces chromatin

condensation and cleavage of DNA into high molecular weight (50kb) fragments when apoptosis is induced (Susin, Lorenzo et al. 1999; Lipton and Bossy-Wetzel 2002; Penninger and Kroemer 2003) (Figure 13). Blocking the AIF pathway attenuates cell death. Microinjection of AIF antisera in the presence of P53 and pan Caspase inhibitor led to a marked reduction of cell death (Cregan, Fortin et al. 2002). AIF is essential to survival in mammals. Knockout of AIF in mouse induces embryonic lethality. AIF null embryonic stem (ES) cells are relatively resistant to apoptosis induced by serum deprivation (Joza, Susin et al. 2001).

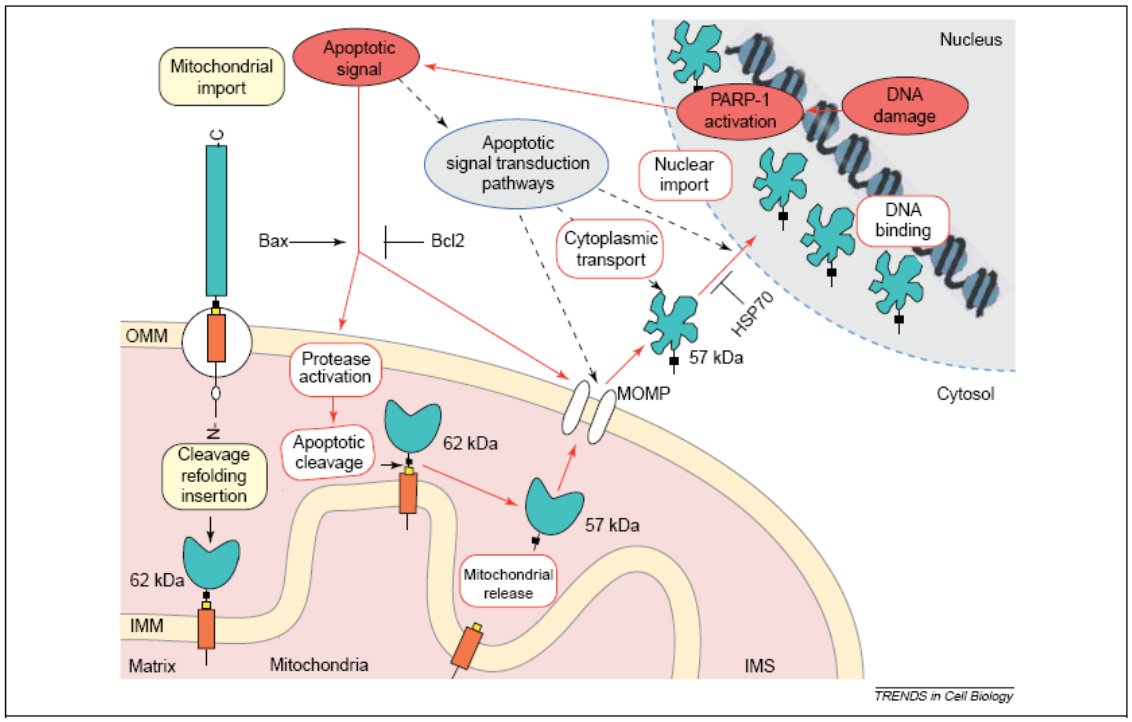


Figure 13. Subcellular localization of AIF. From left to right, the figure shows the import of unprocessed full length AIF into the mitochondrion of a healthy cell. After the cleavage of its N-terminal MLS by a mitochondrial peptidase, AIF is inserted in the inner mitochondrial membrane (IMM), via its N-terminal transmembrane region (orange). The rest of the protein faces the intermembrane space (IMS), where it participates in the regulation of the activity of the respiratory chain. In cells undergoing genotoxic stress, the activation of AIF's nuclear lethal function might be triggered by the PARP1 signaling pathway. The apoptotic release of AIF from mitochondria requires the inducible activity of an unknown protease and mitochondrial outer membrane permeabilization (MOMP). Proteins from the Bcl-2 family regulate these processes. The cytoplasmic transport of AIF is inhibited by the anti-apoptotic Hsp70 protein. Upon translocation to the nucleus, AIF binds to the chromatin and induces its condensation (Modjtahedi et al., 2006).

Mammalian AIF *in vivo* is synthesized as precursor of 67 kDa and resides in the inter-membrane space of mitochondria in a mature form of 62 kDa. Upon induction of apoptosis, processing of mature AIF to an approximately 57 kDa form occurred caspase-independently in the intermembrane space, releasing the processed form into the cytoplasm. Bcl-2 or Bcl-XL inhibited both these events (Otera, Ohsakaya et al. 2005). But there are evidences demonstrating Caspase-dependent AIF release pathway. Addition of recombinant Caspases to purified mitochondria can trigger the release of cytochrome c, and Caspase-2 or Caspase-8-activated bid (tBid) can trigger the AIF release from mitochondria (Cande et al., 2004). AIF nuclear translocation induced by staurosporine or actinomycin D is diminished in the presence of Caspase inhibitor (Arnoult, Parone et al. 2002).

Although most data support a role for AIF in apoptosis, the second function in the protection against oxidative stress has been elucidated. Cells derived from the *harlequin* mouse, a naturally occurring mutant with 80% reduction in AIF levels, exhibit increased sensitivity to oxidative stress (Klein, Longo-Guess et al. 2002). Mice with cardiac and skeletal muscle tissue-specific deletion of AIF exhibit impaired activity and protein expression of respiratory chain complex I, thus, develop severe dilated cardiomyopathy, heart failure, and skeletal muscle atrophy accompanied by lactic acidemia consistent with defects in the mitochondrial respiratory chain (Joza, Oudit et al. 2005). Recently it has been reported that muscle- and liver-specific AIF ablation in mice initiates a pattern of OxPhos deficiency closely mimicking that of human insulin resistance, and contrary to current expectations, results in increased glucose tolerance, reduced fat mass, and increased insulin sensitivity (Pospisilik, Knauf et al. 2007).

EndoG

EndoG localizes to the mitochondria and was identified as an endonuclease activity in the mid 1960s from mitochondria isolated from rat liver and *Neurospora crassa* (Curtis, Burdon et al. 1966; Curtis and Smellie 1966; Linn and Lehman 1966). EndoG is encoded by a nuclear gene and translated in the cytosol including an amino-terminal 48 amino acid domain being the mitochondrial targeting sequence, which is removed when the protein is imported into mitochondria (Cote and Ruiz-Carrillo 1993; Tiranti, Rossi et al. 1995). Levels of Endonuclease G quantified in

mitochondrial extracts *in vitro* have been observed to vary over 200-fold among different rat tissues, with the highest values being found in the heart. The specific activity of EndoG (per mg of mitochondrial protein or per mtDNA circle) seems to reflect the tissue's relative rate of oxygen consumption and, by inference, rate of oxidative injury to mtDNA (Houmiel et al., 1991).

For several years, studies of Endonuclease G focus on understanding what role the enzyme may play in mtDNA metabolism. Most involve the enzyme in mtDNA circles, or mt DNA replication and repair/degradation of damaged mtDNA (Low, 2003).

Recently, evidences support that EndoG is released from mitochondria and contributes to DNA fragmentation during apoptosis (Wang, 2001). The DEF/CAD knockout cells still undergo the nucleosomal DNA cleavage on certain apoptosis stimuli and the DNA fragmentation can not be blocked by Caspase inhibitors. The treatment of mitochondria isolated from embryonic mice fibroblasts lacking DEF with tBid or Bim triggered release of a pool of EndoG present in the intermembrane space and induced a DNA fragmentation which can be blocked by the depletion of EndoG by the anti-EndoG antibody. The immunohistochemistry demonstrated that during the UV treatment the EndoG released from mitochondria eventually localized to nuclear bodies (Li et al., 2001). The same year two independent labs have reported the similar results showing the important role of EndoG in the apoptosis with the isolated mitochondria from mouse liver (van Loo, Schotte et al. 2001) and *Caenorhabditis elegans* (Parrish, Li et al. 2001).

Until now, EndoG knockout mice have been produced from three different labs. Zhang and his colleagues reported that EndoG knock out mice died between embryonic days 2.5 and 3.5 (Zhang, Dong et al. 2003), whereas the other groups independently produced EndoG null mice which can develop to adult. They explain that the first EndoG knock out mice died maybe due to the disruption of the gene that overlaps the EndoG gene. They found that the absence of EndoG does not have a major impact on these apoptosis DNA fragmentation rates or characteristics in the murine B cell and has no effect to the mouse embryonic fibroblasts (MEFs) cell death (Irvine, Adachi et al. 2005; David, Sasaki et al. 2006). However, this two cell types die by Caspase-dependent mechanism. MEFs have Caspases and normally undergo Caspase-dependent DNA fragmentation. MEFs from the DEF45/ICAD-

knockout mice, which have lost the activities of Caspase-3-depedent DNA fragmentation (Zhang et al., 1998), show a Caspase-independent but EndoG-dependent DNA fragmentation (Li et al., 2001). Therefore, EndoG activity is only relevant in Caspase-independent cell death.

1.4 Anti-apoptotic proteins

Besides anti-apoptotic Bcl-2 family proteins, there are several endogenous proteins that can inhibit apoptosis such as IAP (Inhibitors of Apoptosis Proteins) and FLIP (FLICE–like inhibitory protein).

1.4.1 IAPs

Inhibitor of apoptosis (IAP) proteins, a family of anti-apoptotic regulators, can block cell death in response to diverse stimuli through interactions with inducers and effectors of apoptosis. To date IAP family contains seven members: X-linked inhibitor of apoptosis (XIAP/MIHA/hILP/BIRC4/ILP-1); cellular IAP1/Human IAP2 (c-IAP1/HIAP2/MIHB/BIRC2); cellular IAP2/Human IAP1 (c-IAP2/HIAP1/MIHC/API2/BIRC3; Testis-specific IAP (Ts-IAP//hILP2/BIRC8/ILP-2); BIR-containing ubiquitin conjugating enzyme (BRUCE/Apollon/BIRC6); Survivin (TIAP/BIRC5); and Livin (KIAP/ML-IAP/BIRC7) (Hunter, LaCasse et al. 2007).

All IAPs contain one or more 70-80 amino acid baculoviral IAP repeats (BIR) domain by which inhibit Caspase activation (Hinds et al., 1999). Another important domain is carboxy-terminal RING (Really Interesting New Gene). In XIAP, c-IAP1 and c-IAP2, RING has been shown to possess E3 ubiquitin ligase activity leading to protein degradation (Yang, Fang et al. 2000). Only c-IAP1 and c-IAP2 have a CARD domain, it may contribute to interaction with other apoptotic proteins (Martin, 2001). The mechanism by which the IAPs inhibit apoptosis was identified by preventing the subsequent processing of Caspase-3 into its mature subunits. *In vitro* XIAP, c-IAP1 and c-IAP2 could prevent downstream proteolytic processing of procaspase-3,-6,-7 (Deveraux, Takahashi et al. 1997; Deveraux, Roy et al. 1998). Different BIR domain may contribute to inhibit different Caspases (Takahashi, Deveraux et al. 1998; Deveraux, Leo et al. 1999; Huang, Park et al. 2001; Suzuki, Nakabayashi et al. 2001).

Furthermore, the RING domain of IAPs is believed to mediate the ubiquitination and degradation of IAPs, as well as that of their substrates, by which aborts the apoptotic process (Yang, Fang et al. 2000; Suzuki, Imai et al. 2001).

1.4.2 FLIP (FLICE inhibitory protein)

Anti-apoptotic protein FLIP family contains viral–FLICE–inhibitory proteins (v-FLIP) and cellular–FLICE-inhibitory protein (cFLIP). V-FLIP was identified in several heperviruses and the poxvirus MCV and has 6 homologues. C-FLIP has 3 homologues named c-FLIPL; c-FLIPs and c-FLIPR at protein level despite 13 distinct splice variants have been described. All of them have two tandem DED domains. The common anti-apoptotic mechanism is that FLIP can be recruited to the FADD through the DEDs to competitively exclude procaspase-8 from DISC (Krueger, Schmitz et al. 2001) or form heterodimers with Caspase-8 (Golks, Brenner et al. 2005).

Compared with c-FLIPs and c-FLIPR, c-FLIPL contains a Caspase-like domain in C-terminus but lacking the catalytic cysteine residue conserved in Caspases, thereby it is catalytically inactive. Besides the anti-apoptotic role of c-FLIPL, some studies show that it can activate Caspase-8 (Shu, Halpin et al. 1997; Yeh, Itie et al. 2000). The role of c-FLIPL in Caspase-8 inhibition at intermediate expression or Caspase-8 activation at low expression is proposed (Chang, Xing et al. 2002).

CFLIPL could be cleaved by mature Caspase-8 at Asp-376 and /or by procaspase-8 at Asp-196 inducing p22-FLIP and p43-FLIP respectively, by which activate NF-κB pathway (Kataoka and Tschopp 2004; Golks, Brenner et al. 2006). Recently it was demonstrated that JNK activation promotes the proteasomal degradation of c-$FLIP_L$ through the activation of the ubiquitin ligase ITCH, whose catalytic activity is mediated by JNK1-phosphorylation (Chang, Kamata et al. 2006).

1.5 Role of the apoptotic signaling cascade unrelated to cell death

Although the activation of the apoptotic signaling can induce cell death, some functions not directly related to cell death have been observed involving the regulation of cell survival, proliferation, differentiation and inflammation.

Caspases can mediate NF-κB activation, which is a ubiquitous inducible transcription factor that plays key roles in regulating inflammatory and immune responses as well as stress responses. At least two different molecular mechanisms for Caspase-mediated NF-κB activation in mammals have been suggested. A first molecular mechanism of Caspase-mediated NF-κB activation involves the catalytic activity of large prodomain Caspases, such as Caspase-8. Upon T-cell Receptor stimulation, Caspase-8 and FADD are recruited to the CARMA-Bcl10-MALT1 (CMB) complex, by which activate NF-κB (Ruland, Duncan et al. 2003; Su, Bidere et al. 2005). Also in certain cell types (HT1080 human fibrosarcoma, Jurkat T cells, SK-MES-1 human lung squamous carcinoma and A549 human lung carcinoma), RNAi-mediated knockdown of Caspase-8 was shown to impede or delay Apo2L/TRAIL-induced NF-κB activation (Varfolomeev, Maecker et al. 2005). Besides the Caspase-8, short prodomain Caspases may be involved in NF-κB activation via the cleavage of PARP-1. Macrophages from knock-in mice expressing Caspase-resistant PARP-1 show impaired LPS-induced NF-κB-mediated gene activation despite normal binding of NF-κB to DNA. Thus, Caspase-3 and –7 would apparently propagate the transactivation, rather than the nuclear translocation of NF-κB through a PARP-1-mediated mechanism (Petrilli, Herceg et al. 2004). Another mechanism to activate NF-κB may depend on their CARD and DED motif since transient expression of Caspase-1, -2, -8 and -10 are able to activate NF-κB whereas murine Caspase- 9, -11 and -12 are not (Chaudhary, Eby et al. 2000; Lamkanfi, Kalai et al. 2004; Lamkanfi, D'Hondt et al. 2005; Takahashi, Kawai et al. 2006).

Several studies demonstrate an essential role for Caspase-8 in the proliferation of immune cells. Peripheral murine T cells in which Caspase-8 is conditionally deleted are unable to proliferate following TcR activation. More importantly patients with inactivating mutations of Caspase-8 have impaired proliferation of T, B and natural killer (NK) cells. T-cell-specific deletion of Caspase-8 in mice was concluded to recapitulate the immunodeficiency identified in humans with Caspase-8 mutations (Chun, Zheng et al. 2002; Salmena, Lemmers et al. 2003). Humans harboring Caspase-10 point mutations display defective FasL- and TRAIL-induced apoptosis, resulting in the accumulation of lymphoid cells, which is characteristic for type II autoimmune lymphoproliferative syndrome (ALPS type II) (Wang, Zheng et al. 1999). Besides the regulation to proliferation, several reports have demonstrated that

Caspases are involved in the terminal differentiation of a variety of cell types involving enucleation processes such as lens cell differentiation, erythrocyte and platelet formation and the terminal differentiation of keratinocytes. Caspase-3 activity is apparently required for the maintenance of lens transparency, since Caspase-3-/- mice exhibit marked cataracts at the anterior lens pole (Zandy, Lakhani et al. 2005). Another report established a role for Caspase-3 in skeletal muscle differentiation: primary myoblasts from Caspase-3 knockout mice displayed a severe lack of myotube and myofiber formation and a reduced expression of muscle-specific genes (Fernando et al., 2002). In contrast to these roles of Caspases as positive regulators of cell differentiation, constitutive Caspase-3 activity in immature dendritic cells was found to block dendritic cell maturation and cell-surface expression of peptide-loaded major histocompatibility complex (MHC) class II molecules (Santambrogio et al., 2005).

Cell motility also can be regulated by non-apoptotic signaling. Caspase-8-deficience and CrmA-overexpression hamper cell migration in MEF cells (Helfer et al., 2006). In addition, embryonic Caspase-8 deficient mice fail to assemble a functional circulatory system, which may attribute to a defect in endothelial cell migration (Varfolomeev, Schuchmann et al. 1998).

Interestingly, Caspase-8, FADD, c-FLIP knock out and Caspase-3/7 double knock out mice show similar heart phenotype including dilation of the atria and disorganization and noncompaction of the ventricular musculature (Varfolomeev, Schuchmann et al. 1998; Yeh, Pompa et al. 1998; Yeh, Itie et al. 2000; Lakhani, Masud et al. 2006). It indicates that these proteins play an important role in heart development not related to cell death function. Furthermore, it has been reported that skeletal muscle differentiation depends on the activity of the key apoptotic protease, Caspase-3. Caspase-3-/- myoblasts display a differentiation deficit. Peptide inhibition of Caspase-3 activity or homologous deletion of Caspase-3 leads to dramatic reduction in both myotube/myofiber formation and expression of muscle-specific proteins in C2C12 cell line (Fernando et al., 2002). Caspase-9 is responsible for the activation of Caspase-3 in differentiating C2C12 cells. Reduction of Caspase-9 levels, using a shRNA construct or overexpression of Bcl-XL, prevented Caspase-3 activation and inhibited myoblast fusion. Myosin-heavy-chain expression, which accompanies myoblastic differentiation, was not Caspase-dependent. These data

suggest that the mitochondrial pathway is required for differentiation; however, the release of cytochrome *c* or Smac (Diablo) could not be detected, raising the possibility of a novel mechanism of Caspase-9 activation during muscle differentiation (Murray et al., 2008).

1.6 Regulation of apoptotic protein activity

1.6.1 Level of regulation

Transcription. Another mechanism can be induced by transcription factor. For example, NOXA (Oda, Ohki et al. 2000) and PUMA (Nakano and Vousden 2001; Yu, Zhang et al. 2001) are induced by the tumor suppressor p53 in response to DNA damage.

Translation. The translation not only can be regulated on the transcriptional level, but also can happen on post-transcriptional level. For example, Apaf-1 translation can initiate by internal ribosome entry (IRES) mechanism without affecting the transcript (Mitchell et al., 2001).

Post-translation. Post-translational regulation can also be one of important mechanisms.

Release. Bim and Bif can release from the microtubule and actin cytoskeletons respectively to be activated (Puthalakath, Huang et al. 1999; Puthalakath, Villunger et al. 2001). Bad dephosphorylation results in liberty to the 14 to 3-3 protein and translocation to the mitochondria (Zha, Harada et al. 1996).

Cleavage. Caspases are cleaved by itself or initiator Caspases to get activated. Bid is cleaved by Caspase-8 (Li, Zhu et al. 1998; Luo, Budihardjo et al. 1998; Gross, Yin et al. 1999) , calpain (Chen et al., 2001a), and granzyme B (Barry, Heibein et al. 2000, Sutton, Davis et al. 2000) to be truncated Bid exposing BH3 domain.

Phosphorylation. BAD is activated by loss of phosphorylation in response to growth-factor deprivation (Zha et al., 1996). FADD, especially its phosphorylation, is involved in lymphocyte proliferation and activation as well as apoptosis (Hua et al., 2003; Walsh et al., 1998). Akt phosphorylates Caspase-9 at Ser-196 by which overexpressing Akt block cytochrome C-mediated caspase 9 activation in vitro (Cardone et al., 1998). Phosphorylation of Bcl-2 by JNK occurs at the ER membrane. This modification regulates the antiapoptotic activity of Bcl-2 negatively, correlating

with a decreased binding to BH3-only proteins and increased ER calcium content (Bassik et al., 2004).

Ubiquitination. Ubiquitination-mediated degradation and change in activity regulate many molecules of the cell death. Proapoptotic Bcl-2 family members Bax, Bik and tBid are degraded by the ubiquitination and proteasome systems (Breitschopf et al., 2000; Li and Dou, 2000; Marshansky et al., 2001). Studies also show that the interactions between XIAP and caspases-proteases participate in the ordered disassembly of the cell during apoptosis. In addition, XIAP can function as an E3 ligase to catalyze the ubiquitination of substrate proteins (Galban and Duckett, 2009).

1.6.2 Focus on E2F and PTB

Diverse mechanisms have been shown to regulate apoptotic protein activity. E2Fs and PTBs, two potential candidates, have been suggested to be involved in apoptotic gene expression at the transcriptional and post-transcriptional levels, respectively.

1.6.2.1 E2Fs

Members of the E2F family of transcription factors are downstream effectors of the tumor suppressor pRB and are considered to have a pivotal role in controlling cell-cycle progression. E2Fs can both activate and also repress gene expression. Furthermore, E2Fs function in a wide range of biological processes, including DNA replication, mitosis, the mitotic checkpoint, DNA-damage checkpoints, DNA repair, differentiation, development and apoptosis (Ren, Cam et al. 2002; Bracken, Ciro et al. 2004; Cam, Balciunaite et al. 2004; Dimova and Dyson 2005).

In mammals, the E2F family comprises eight genes (E2F1–8), which give rise to nine distinct proteins. These include E2F3a and E2F3b, which are generated by the use of alternative promoters (DeGregori and Johnson, 2006). E2F-family members have been categorized into subfamilies based on their transcriptional activity, structure and interaction with pRB-family members, p107 and p130. E2F1, E2F2 and E2F3a, which interact only with pRB, constitute one subfamily and are often referred to as the ‘activator E2Fs’, because they are believed to function mainly in activating

gene expression. However, this classification is probably an over-simplification because DNA microarray studies show that activation of the so-called activator E2Fs leads to repression of almost as many genes as they activate (Muller, Bracken et al. 2001; Ma, Croxton et al. 2002; Polager, Kalma et al. 2002; Young, Nagarajan et al. 2003). E2F4–8 function mainly in repression of gene expression and are often referred to as the 'repressor E2Fs'. Some genetic models have shown the effect of E2Fs (Table 3).

Genotype	Cell type or tissue affected	Affect on proliferation or apoptosis	Phenotypic consequences
E2F1-/-	T cells	reduced proliferation	
	thymocytes	decreased apoptosis	defective negative selection
	thymocytes and cultured tumor cells	decreased chemotherapeutic induced apoptosis	
	keratinocytes	increased UVB induced apoptosis with decreased DNA repair	
E2F2-/-	T cells	increased antigen dependent proliferation	
E2F1-/-E2F2-/-	T cells	increased antigen dependent proliferation	
	T cells	decreased homeostatic proliferation	T cell lymphopenia
	hematopoietic progenitors	impaired S phase progression	defective hematopoiesis, anemia and leukopenia
	exocrine pancreas	increased endoreduplication	polyploidy, exocrine degeneration and diabetes
E2F3-/-	MEFs	decreased S phase entry	
E2F1-/-E2F2-/-E2F3-/-	MEFs	arrest throughout cell cycle	
E2F4-/-E2F5-/-	MEFs	impaired cell cycle arrest mediated by $p16^{Ink4a}$ or inhibition of Ras	

Table 3. Alterations in proliferation and apoptosis resulting from targeted E2F mutations in mice. Effects of E2F disruptions on proliferation or apoptosis are summarized. While disruptions of E2F3, E2F4, E2F5 and E2F6 genes have clear effects on development or tissue function in mice, it is not clear how possible cell autonomous effects of the loss of these E2Fs on proliferation might link to developmental defects, and thus these phenotypes are not shown. While connections between altered proliferation/apoptosis and phenotypes of knock-out mice are somewhat speculative, the last column does provide suspected connections where logical associations can be drawn (DeGregori and Johnson, 2006).

E2Fs can promote or inhibit apoptosis. Using high-density oligonucleotide arrays to identify genes in which expression changed in response to activation of E2F1, E2F2, and E2F3, showing that the E2Fs regulate a number of genes involved in apoptosis, differentiation, and development (Muller, Bracken et al. 2001). E2F dependent, and in some cases E2F1 specific, regulation of a number of pro-apoptotic target genes, including regulators of p53, p73, Bcl2 family members, Caspase-8 and Apaf-1, appear to underlie transcription-dependent apoptosis induction by E2F1

(Irwin, Marin et al. 2000; Stiewe and Putzer 2000; Moroni, Hickman et al. 2001; Nahle, Polakoff et al. 2002; Cao, Xia et al. 2004; Hershko and Ginsberg 2004; Hershko, Chaussepied et al. 2005). Moreover, Nahle et al. found that deregulation of E2F by adenovirus E1A, loss of Rb or enforced E2F-1 expression results in the accumulation of Caspase proenzymes through a direct transcriptional mechanism. Increased Caspase levels seem to potentiate cell death in the presence of p53-generated signals that trigger Caspase activation (Nahle, Polakoff et al. 2002). In primary rat cardiomyocytes adenoviral overexpression of E2F-1 resulted in an increase in the expression of key cell cycle activators and apoptosis in 90% of the cells (von Harsdorf, Hauck et al. 1999). E2F-/- mice develop and reproduce normally, but exhibit a defect in T lymphocyte development leading to an excess of mature T cells due to a maturation stage-specific defect in thymocyte apoptosis (Field, Tsai et al. 1996). Interestingly it seems that in the E2F family E2F1 is crucial for E2F-dependent apoptosis. E2F1 overexpression induces cells to undergo apoptosis, despite the fact that at least two other E2F family members, E2F2 and E2F3, are equally capable of inducing S phase (DeGregori et al., 1997). Ectopic expression of E2F3 results in apoptosis in both primary mouse fibroblasts and transgenic mice. Apoptosis induced by E2F3 is associated with the accumulation of E2F1 and, strikingly, E2F3-induced apoptosis is dependent on E2F1 (Lazzerini Denchi and Helin, 2005).

1.6.2.2 PTB

Polypyrimidine tract binding proteins (PTBs), also known as hnRNP I, were first identified as one of the components of the pre-mRNA splicing machinery, where its target RNA is the U/C-rich region of introns (Patton, Mayer et al. 1991). Structurally, PTB is composed of four RRM domains with interdomain linker regions and an N-terminal extension containing both nuclear localization and export signals. PTB exists in two major alternatively spliced isoforms, termed PTB1 and PTB4, which arise from skipping or inclusion, respectively, of exon 9, which encodes a 26-amino acid insert. A minor isoform, PTB2, is produced by inclusion of exon 9 using an internal 3'-splice site, giving a 19-amino acid insert (Gil, Sharp et al. 1991; Ghetti, Pinol-Roma et al. 1992). In addition mammals have two other paralogues, nPTB

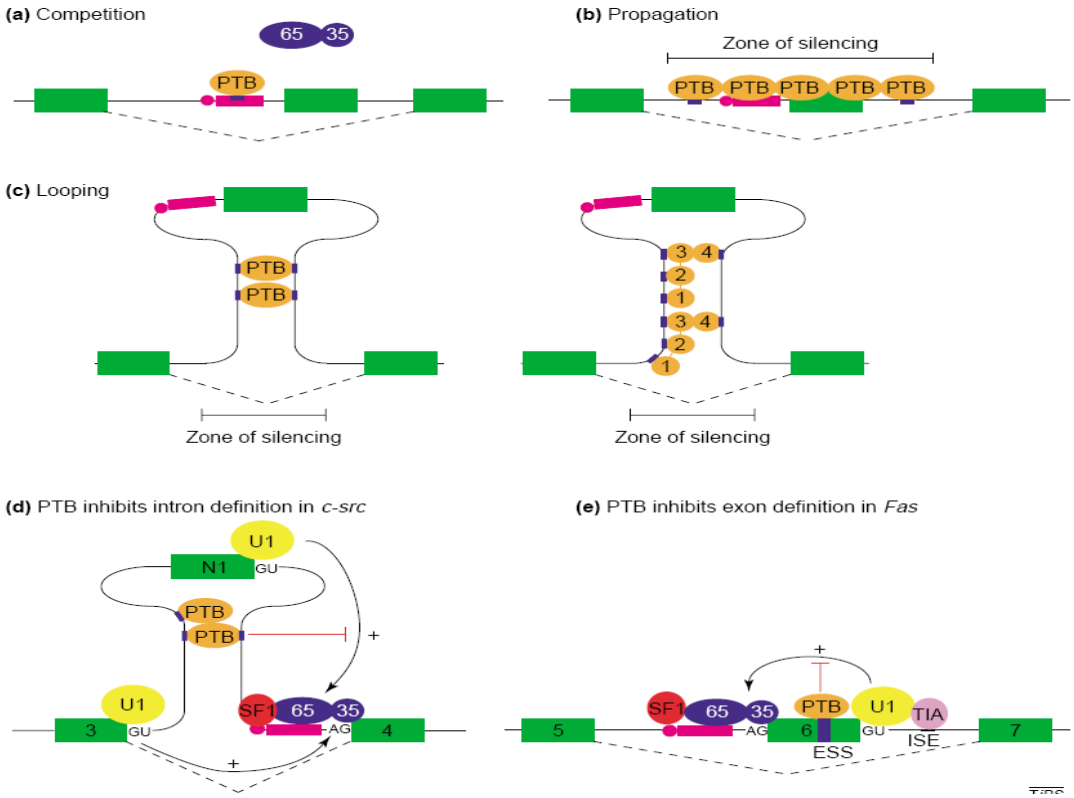


Figure 14. Models of PTB repression. Exons are shown as green boxes, introns as black lines, branch-point sequences and polypyrimidine tracts as pink circles and rectangles, respectively, and PTB-binding silencers as small blue boxes. (a) Competitive binding of PTB and U2AF65 to a regulated PPT. (b) Propagation of PTB molecules between high affinity sites (the PTB-binding silencers) leads to masking of an exon. (c) Looping of exons as a result of PTB–PTB interactions between molecules bound to sites flanking the exon. A model suggested by Oberstrass et al. in which the four RRM domains of PTB are shown. Looping occurs as a direct result of the relative orientation of RRMs 3 and 4, which interact with each other using their 'dorsal' surfaces on the opposite side from their RNA-binding b-sheet faces. Cooperative binding of a second monomer can arise as a result of the first bound monomer, bringing additional PTB-binding sites into closer spatial proximity. Simple blockage of splice sites or auxiliary elements in (a)–(c) is thought to prevent assembly of early splicing complexes. (d) Model for PTB interference of intron definition between c-src N1 exon and exon 4. Cooperative binding of PTB to silencer elements flanking the N1 exon does not prevent U1 snRNP binding to the 50 splice site of N1. Instead, the bound PTB prevents U1 snRNP at this splice site from making productive cross-intron interactions with U2AF at the PPT of constitutive exon 4. By contrast, U1 snRNP at the upstream 50 splice site of constitutive exon 3 remains free to form cross-intron interactions with U2AF. (e) Model for PTB interference with exon definition of Fas exon 6. The protein TIA-1 promotes exon definition by binding to an intron-splicing enhancer (ISE) and promoting U1 snRNP binding. PTB binds to an exon-splicing silencer (ESS) in exon 6 and inhibits the activation by U1 snRNP of U2AF65 binding at the PPT, without affecting binding of U1 snRNP itself (Spellman and Smith, 2006).

(Markovtsov, Nikolic et al. 2000; Polydorides, Okano et al. 2000) and ROD1 (Yamamoto, Tsukahara et al. 1999), which show a more restricted expression in neurons and haematopoietic cells respectively. A fourth paralogue, smPTB, is expressed at high levels in smooth muscle cells of rats and mice, but is restricted to rodents (Gooding et al., 2003).

Polypyrimidine tract binding protein has been found to be a repressive splicing regulator (Spellman and Smith, 2006). It acts as a splicing repressor of exons in a number of genes including c-src, g-GABAA receptor g2, α- and β-tropomyosins, fibronectin, α-actinin, and fibroblast growth factor receptors (FGFR)-1 and -2 (Mulligan, Guo et al. 1992; Lin and Patton 1995; Singh, Valcarcel et al. 1995; Ashiya and Grabowski 1997; Chan and Black 1997; Perez, Lin et al. 1997; Gooding, Roberts et al. 1998; Southby, Gooding et al. 1999; Wagner, Carstens et al. 1999; Zhang, Liu et al. 1999; Carstens, Wagner et al. 2000; Jin, McCutcheon et al. 2000). Also PTB can repress itself exon 11 including and this alternative skipping of PTB leads to an mRNA that is removed by nonsense-mediated mRNA decay in HeLa cells (Wollerton, Gooding et al. 2004) (Figure 14). In addition PTB also play roles in pre-mRNA 3'-end processing (Castelo-Branco et al., 2004), stability (Hamilton, Genin et al. 2003) and localization (Cote, Gautreau et al. 1999).

Besides to these functions, PTB activates IRES-driven translation (Kaminski, Hunt et al. 1995; Hunt and Jackson 1999). This additional ability of PTB was revealed by studies of picornaviruses. The genomic RNA of these viruses is translated from internal ribosome entry sites (IRES) by mechanisms distinct from those governing the cap-dependent initiation of translation of most eukaryotic mRNAs. PTB was found associated with many picornavirus IRES (Jackson and Kaminski, 1995), and depletion/reconstitution experiments demonstrated that PTB stimulates translation initiated from at least two of them (Kaminski, Hunt et al. 1995; Niepmann 1996). Multiple IRES have subsequently been found on different viral mRNAs, and in cellular mRNAs (Macejak and Sarnow 1991; Nanbru, Lafon et al. 1997; Johannes, Carter et al. 1999). The presence of IRES elements in viruses provides them with the advantage to hi jack the translational machinery of the host cell to favor the expression of foreign transcripts. Most of the cellular IRES have been shown to function preferentially when cap-dependent translation is physiologically impaired. Consistent with this concept, IRES elements were active

during γ-radiations (Holcik, Lefebvre et al. 1999), hypoxia (Stein et al., 1998) or amino acid starvation (Fernandez et al., 2001). This process requires the direct recruitment of ribosomes to a complex RNA structural element (an internal ribosome entry segment, IRES) that is generally formed in the 5' untranslated regions (UTR) of the mRNA; IRES-mediated translation requires or is facilitated by IRES trans-acting factors (ITAFs) such as PTB. Apaf-1 translation is solely initiated by internal ribosome entry because the 5' untranslated region (UTR) of Apaf-1 is long, G-C rich and has the potential to form complex secondary structures (Coldwell et al., 2000). And the only situation where a small increase in Apaf-1 IRES function was observed was following genotoxic stress (Subkhankulova, Mitchell et al. 2001) (Figure 15). IRES translation of Apaf-1 has been shown to be functionally dependent, both in vitro and in vivo, on PTB (Mitchell, Brown et al. 2001; Mitchell, Spriggs et al. 2003). Thus, it is possible that PTB induced IRES could be required for the expression of Apaf-1 during development.

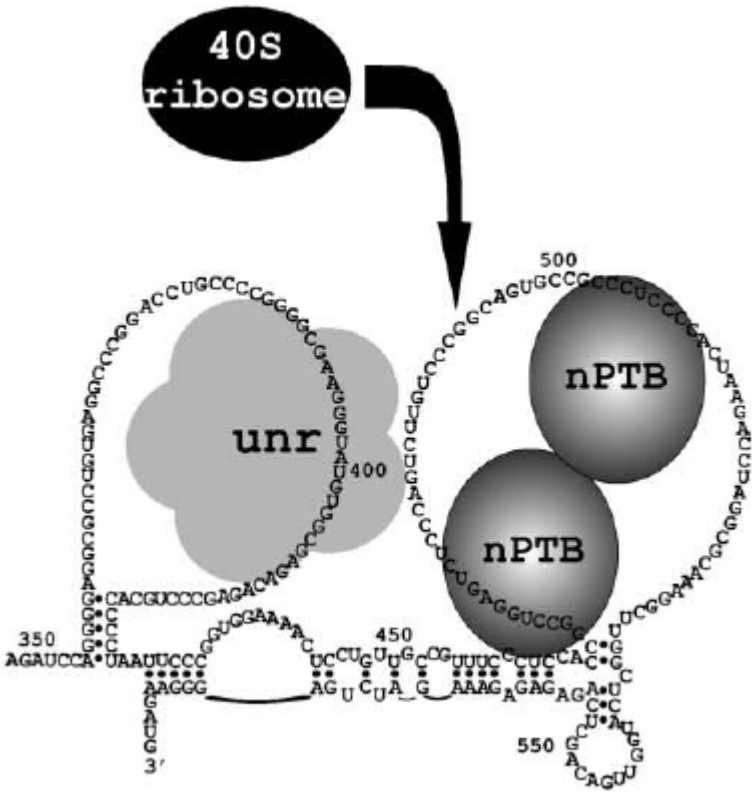


Figure 15. A Model for the Binding of unr and nPTB to the Apaf-1 IRES during Ribosome Recruitment (Mitchell, Spriggs et al. 2003)

2. The heart

2.1 Architecture and function of the heart

The heart is a pumping organ that supplies blood to the body. The human heart has four-chambers, including Right atrium, Right ventricle, Left atrium and Left ventricle. The two atria are thin-walled chambers that receive blood from the veins. The two ventricles are thick-walled chambers that pump blood out of the heart. Heart links with arteries and veins to receive deoxygenated blood and pump oxygenated blood out. Deoxygentated blood returns to the heart via the vena cava. This blood is sent to the lungs along the pulmonary arteries to collect oxygen. It returns through the pulmonary veins to the left atrium. The left ventricle which receives blood from the left atrium pumps the oxygenated blood to the aorta, the main artery from the heart, which carries oxygenated blood to the body and head. The heart itself also receives the oxygenated blood through coronary arteries.

The heart fluid flows always in one direction without exception. The right atrium receives deoxygenated blood from systemic veins; the left atrium receives oxygenated blood from the pulmonary veins. A set of valves contribute to keep the correct direction of the flow. The right atrioventricular valve is the tricuspid valve. The left atrioventricular valve is the bicuspid, or mitral, valve. The valve between the right ventricle and pulmonary trunk is the pulmonary semilunar valve. The valve between the left ventricle and the aorta is the aortic semilunar valve. When the ventricles contract, atrioventricular valves close to prevent blood from flowing back into the atria. When the ventricles relax, semilunar valves close to prevent blood from flowing back into the ventricles (Zaret et al., 1992).

The outflow tract (OFT) is abnormal in many congenital heart defects. Apoptosis has been shown to be involved in the correct formation of the OFT. Chicken embryos (stages 19–38; ED4–10) stained with a fluorescent supravital lysosomal dye (LysoTracker Red; LTR) revealed the three-dimensional relationship between structural changes and apoptosis. The LTR staining peaked in the OFT myocardium at stages 27–32. While LTR stained under both the pulmonary artery and the aorta, it was most prevalent in the subaortic myocardium before its elimination. Furthermore, LTR staining was most abundant in the myocardium under intensely cytokeratin-positive, thick epicardium. It indicates that temporally and spatially restricted

apoptosis in the OFT allows the aorta and pulmonary artery to dock at the appropriate angle and level with the proper ventricle (Schaefer et al., 2004).

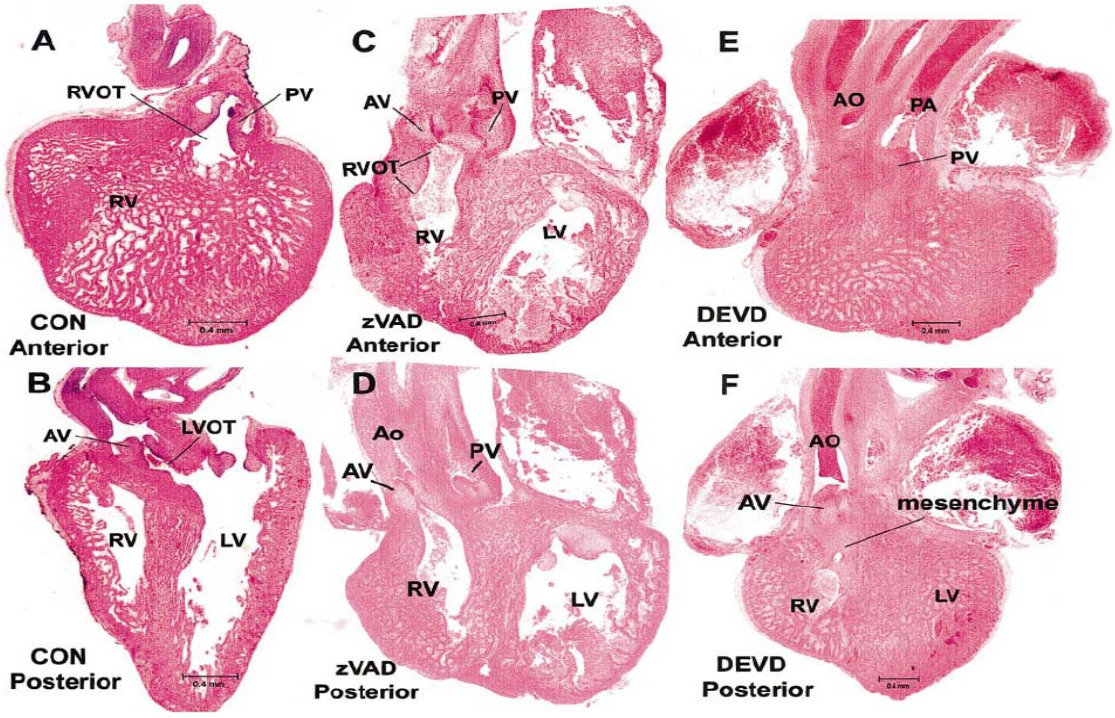


Figure 16. Histology of hearts treated with peptide Caspase inhibitors. Sections are shown from stage-35 control (A, B), zVAD-fmk- (C, D), and DEVD-CHO-CP (E, F)-treated hearts. Frontal sections are shown to best reveal the relationships of the great vessels with the ventricles. One anterior and one posterior section are shown for each heart to reveal the origin of the PA and Ao, respectively. (A, B) A control heart showing the myocardial (infundibular) connection of the PA to the RV (labeled RVOT) anteriorly and coursing to the left (A), and the direct connection of the Ao to the LV posteriorly and coursing to the right (B). (C) A zVAD-fmk-treated heart showing a long and thick RVOT extending above the base of the ventricle and connecting to the orifices for both the PA and Ao (C). The RV-free wall is also markedly hypertrophied. The semilunar valves are globular and in continuity via a persistent mesenchyme. (D) More posteriorly, the rightward vessel, the AO, can be seen connecting to the RV. The AVs and PVs are evident in the same plane and at the same vertical level, and are more globular then normal. (E, F) A DEVD-CHO-CP-treated heart showing a markedly hypercellular OFT comprised of both myocardium and mesenchymal tissue. The Ao also connects to the RV, and both AV and PV are globular and poorly developed. There is also marked increase in the trabeculae that fill the ventricles. Clotted blood fills the vessel lumens. RVOT, right ventricular outflow tract; LVOT, left ventricular outflow tract; PV, pulmonic valve; AV, aortic valve. Bar, 0.4 mm (A–F) (Watanabe et al., 2001).

Using Caspase inhibitors zVAD-fmk or DEVD-cho or recombinant adenovirus expressing the X-linked inhibitor of apoptosis protein (XIAP) to chick embryos at Hamburger and Hamilton (HH) stages 15–20 (looped heart), it induced the abnormal persistence of a long infundibulum (OFT myocardial remnant) beneath the great vessels at HH stage 35, indicating failure of OFT shortening whereas the normal hearts have developed a dual circulation. In some instances, the infundibulum connected both great vessels to the right ventricle in a side-by-side arrangement with transposition of the aorta, indicating a failure of rotation of the OFT, and modeling human congenital double outlet right ventricle. Defects were also observed at other sites in the heart where apoptosis is prevalent, such as in the formation of the cardiac valves and trabeculae. These results demonstrate that elimination of OFT cardiomyocytes by apoptosis is necessary for the proper formation of the ventriculo-arterial connections, and suggest apoptosis as a potential target of teratogens and genetic defects that are associated with congenital human conal heart defects (Watanabe et al., 2001) (Figure 16).

2.2 Cardiac morphogenesis

Cardiac myocytes are derived from the mesodermal germ layer, and the position of their progenitor cells can be mapped within the primitive ectodermal layer prior to gastrulation. The bilaterally-arranged progenitors migrate through the node and primitive streak at gastrulation, and move to the anterior and anterior / lateral aspects of the embryo to form the so-called paired heart fields which then converge anteriorly over the foregut lip to form the cardiac crescent. Heart field cells are defined as those that have cardiac developmental potency when explanted and placed into culture. In lower vertebrates such as amphibia, heart fields may include cells that do not ultimately contribute to the heart, but can do so in a regenerative mode if definitive precursors are removed or damaged. In a progression associated with foregut pocket formation, heart progenitor cells move ventrally and fuse at the midline to create the linear heart tube. This consists of an inner endothelial tube shrouded by a layer of myocardium that remains attached to the ventral aspect of the foregut. The elongating heart tube then largely loses its connection to the foregut and adopts a rightward spiral in a process termed cardiac looping morphogenesis. It is during looping that the primitive cardiac chambers become evident as a pattern of

swellings and constrictions along the heart tube. Looping is guided by an embryonic left / right axial pathway that determines the rightward direction of ventricular bending and distinct morphological identities of left and right atria. At the completion of looping (around E12.5 in the mouse), the heart has assumed a form that closely resembles that of the adult organ. There is extensive remodeling of the internal structures of the heart during looping, with septation and valvulo genesis completing the separation and connectivity of the chambers (Solloway and Harvey, 2003) (Figure 17).

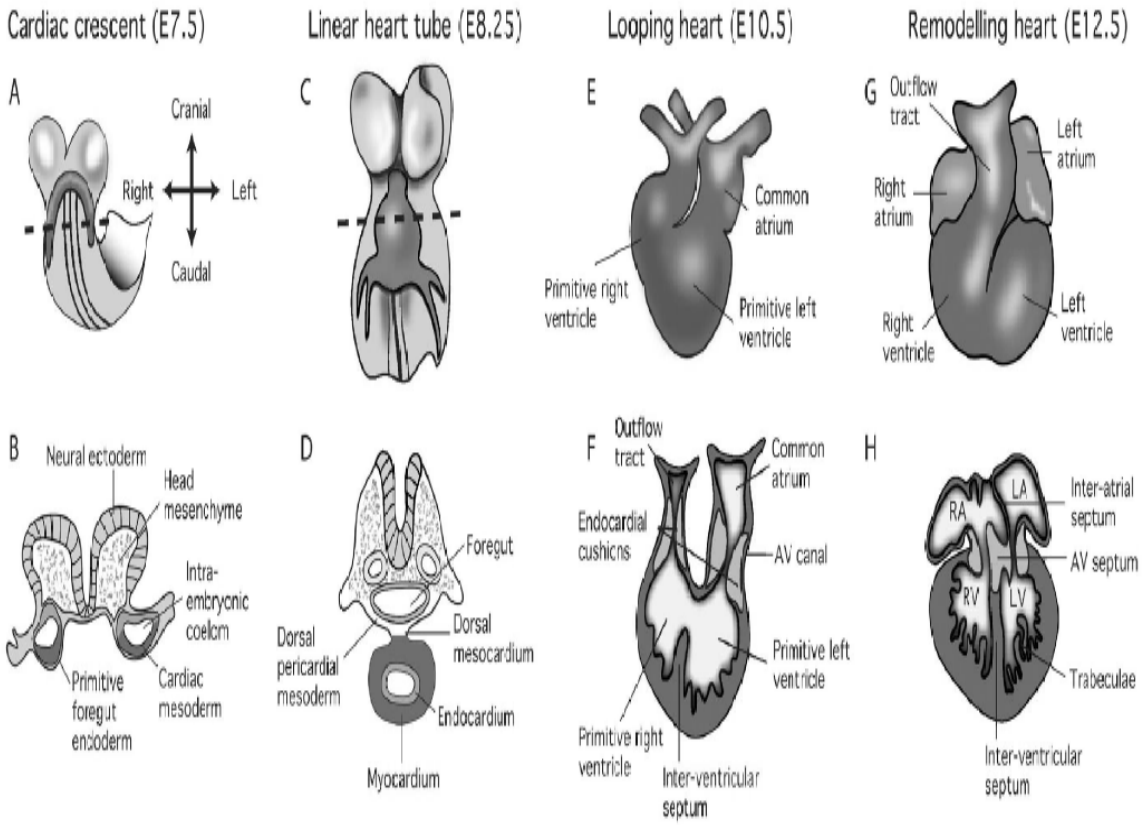


Figure 17. Heart development. Stylised pictures (above) and sections (below) depict the major transitions in early mammalian heart development. Dark shading represents myocardial tissue. AV, atrioventricular; LA, left atrium; LV, left ventricle (Harvey et al., 2002).

2.3 Proliferation, differentiation of cardiomyocytes in the postnatal period

Most of heart is made up of contractile muscle cell, as known as cardiomyocytes. Cardiomyocytes constitute approximately 75% of the total volume of the myocardium (Brilla et al., 1991; Zak, 1973). Cardiomyocytes occupy most normal myocardial tissue volume, but they are the minority (only 30%) in terms of cell numbers. In the human heart, for example, myocytes account for only half of the cells at birth, and this share drops to about one third within 2 months of postnatal development, mainly because of continued fibroblast proliferation (Adler et al., 1981).

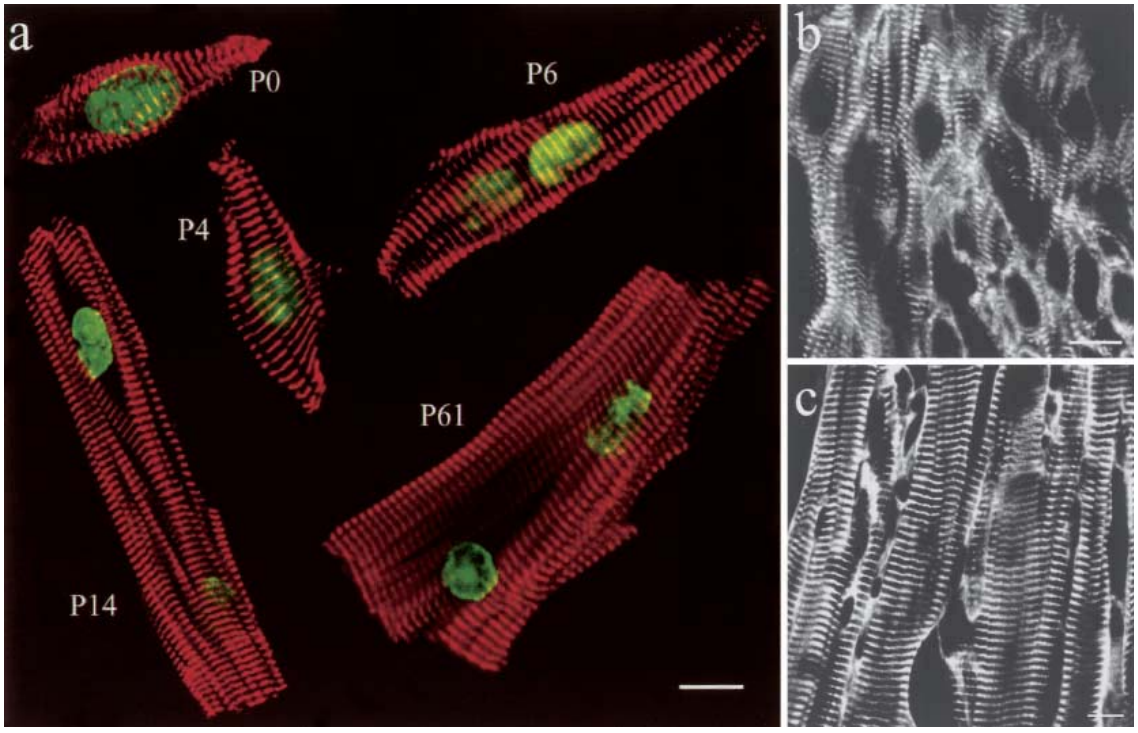


Figure 18. Fixed freshly isolated mouse cardiomyocytes of different ages (*P0*, *P4*, *P6*, *P14*, *P61*) labeled with an anti-myomesin antibody (in red) and Pico Green (in *green*) as a nuclear stain (**a**).Myomesin is located in the M-band. During the postnatal phase amorphological change from small spindle-shaped cells (P0, P4) to large rod-shaped cells occurs. Additionally from P6 onwards most of the cardiomyocytes contain two nuclei. Cryosections from newborn (**b**) and adult (**c**) heart stained with anti-myomesin antibodies, demonstrating similar shape differences as seen in the freshlyisolated cardiomyocytes. *Bar* 10 μm (Leu et al., 2001)

Embryonic cardiac myocytes undergo replication while expressing tissue-specific structural genes (Zak, 1974). After birth, cardiomyocytes soon lose their ability to proliferate. There are three phases in rat heart development after birth (Figure 18) :(1) hyperplasic phase, which last until postnatal day 4 (P4). In this phase cardiomyocytes keep their ability of proliferation. The cell is spindle-shaped and only has one nucleus. (2) a transitional stage between P5 and P15. In this stage cell division stop and DNA synthesis continues until the hypertrophy phase (Claycomb, 1975; Claycomb, 1979; Yoshizumi et al., 1995) (3) hypertrophy phase which is from P15 onwards. In this phase the cells become a rod shape and contain two nuclei (Clubb and Bishop, 1984; Leu et al., 2001; Li et al., 1996; Oparil et al., 1984) (Figure 19).

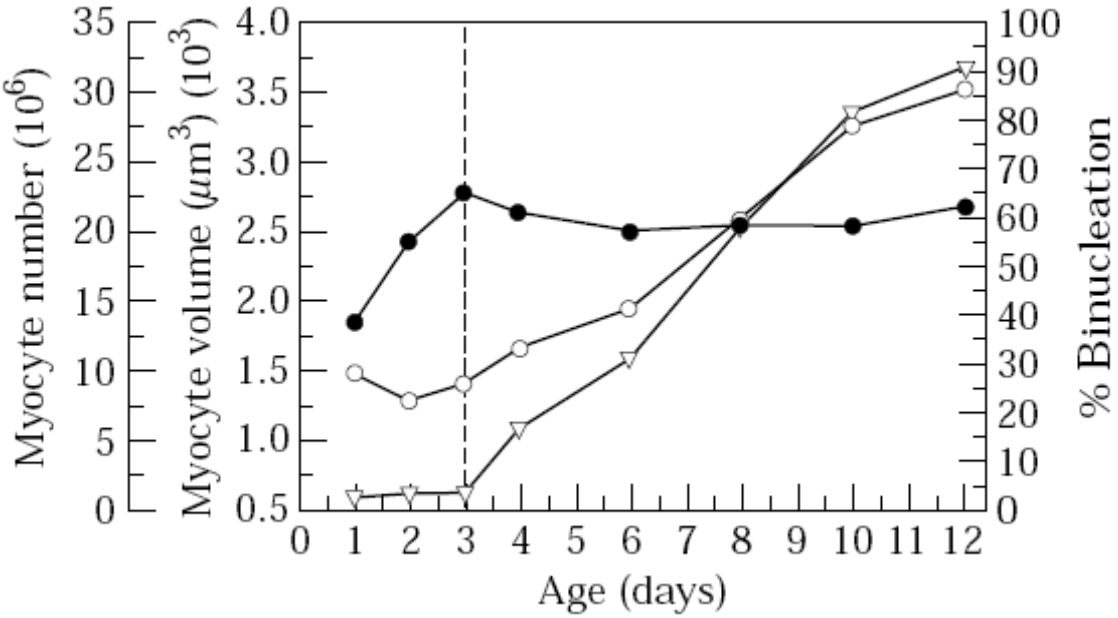


Figure 19. Changes in myocyte number per heart, myocyte volume (○) and per cent nucleation (▽) in contractile cells enzymatically isolated from the hearts of neonatal rats at 1, 2, 3, 4, 6, 8, 10, and 12 days of age. Myocyte number (●) reaches a peak at 3 days of age, remaining unchanged thereafter. At this time point (3–4 days of age) myocyte volume progressively increases while the percentage of myocytes that are binucleated also increases indicating the cessation of myocyte cellular hyperplasia, continuation of nuclear division, and the induction of myocyte hypertrophic enlargement. Each point represents the average value from at least ten hearts. See Table 1 for mean values±S.D (Li et al., 1996).

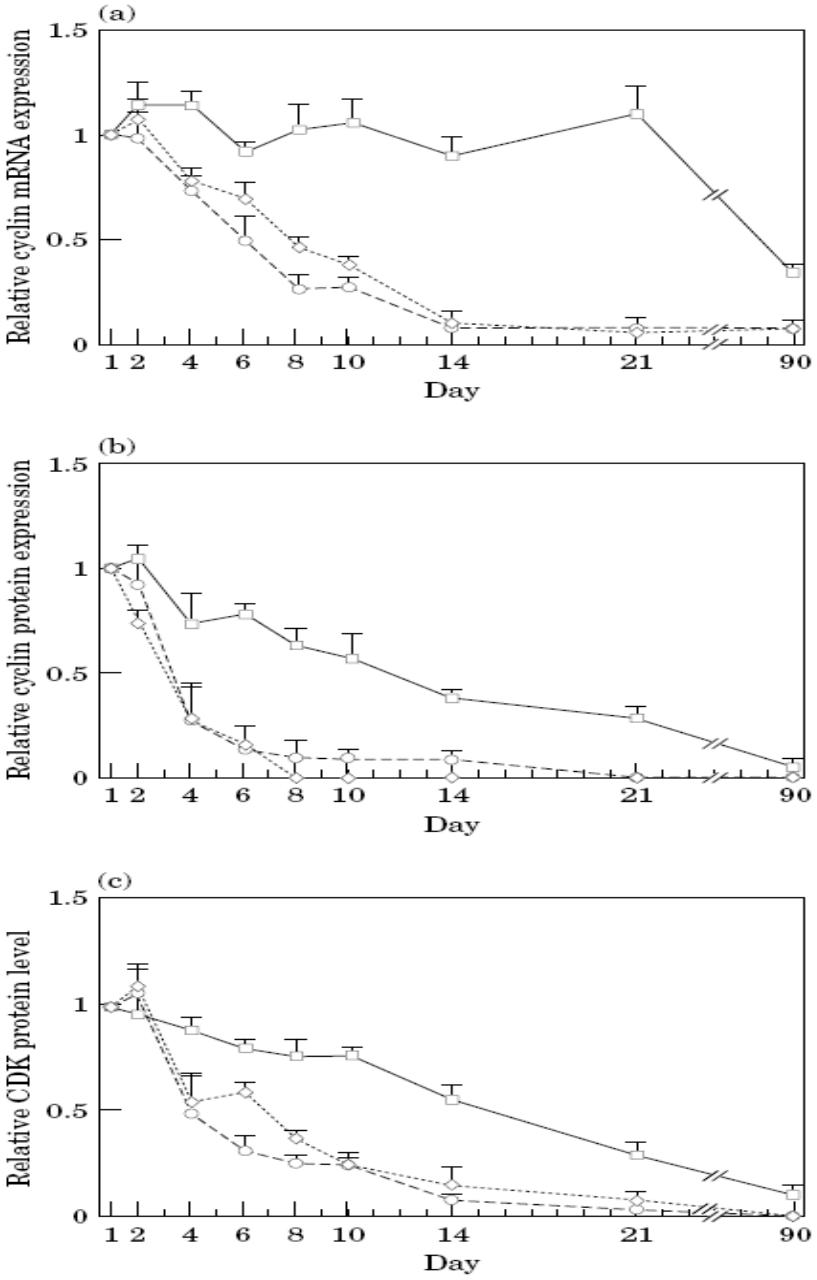


Figure 20. Densitometric analysis of signals of cyclin mRNAs (a), cyclin proteins (b) and CDKs (c) in cardiomyocytes during the neonatal period. From Northern blot results, signals (upper bands for cyclin A and middle bands for cyclin B were assessed) were normalized by corresponding GAPDH signals. Density of each cyclin and CDK in day 1 was presented as 1. Data are presented as mean ± standard error of arbitrary ratios from three results of each experiment. Each line indicates changes of relative density of cyclins and CDKs. The legends of postnatal stages are the same as shown in Figure 1. (a) and (b): (□) cyclin D1; (◇) cyclin A; (O) cyclin B. (c): (□) CDK4; (◇) CDK2; (O) cdc2 (Kang and Koh 1997).

During the transition from hyperplasia to hypertrophy expression and activity of cell cycle regulatory molecules of myocytes change (Poolman and Brooks, 1998) (Figure 20). The percentage of myocytes found in the S phase of the cell cycle decreased significantly during the transition from hyperplasia to hypertrophy (5.5, 3.5, 2.3 and 1.9% of cells in 2-, 3-, 4- and 5-day-old myocytes, respectively, $P<0.05$), concomitant with a significant increase in the percentage of G0/G1 phase cells. At the molecular level, the expression and activity of G1/S and G2/M phase acting cyclins (cyclin A and cyclin E) and CDKs (cdc2 and CDK2) were downregulated significantly during the transition from hyperplasia to hypertrophy, whereas the expression and activity of G1 phase acting cyclins (cyclin D2 and cyclin D3) and CDKs (CDK4 and CDK6) were upregulated significantly during this transition. In addition, p21CIP1- and p27KIP1- associated CDK kinase activities remained relatively constant when histone H1 was used as a substrate, whereas phosphorylation of the retinoblastoma protein was upregulated significantly during the transition from hyperplasia to hypertrophy.

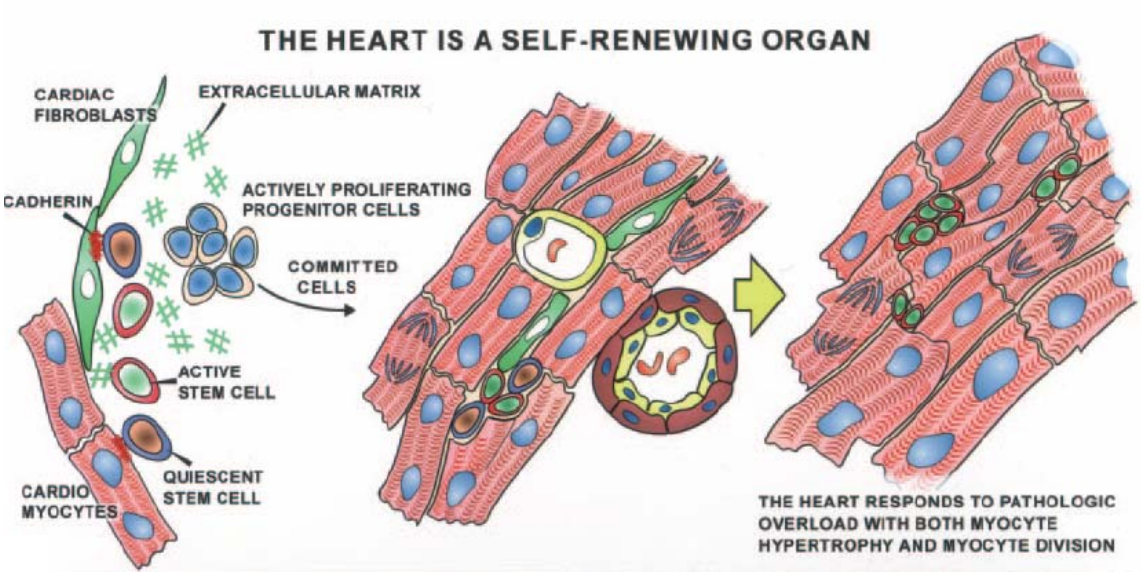


Figure 21. New paradigm of the heart. Cardiac niches contain stem cells, which, after activation, give rise to myocytes and vascular structures (Anversa, Kajstura et al. 2006).

Contrary to the dogma that the heart is a postmitotic organ incapable of regeneration, recently some data suggest that heart is a self-renewing organ. Myocyte

replication, karyokinesis, cytokinesis, and foci of spontaneous myocardial regeneration have been documented in the human heart (Urbanek et al., 2003; Urbanek et al., 2005). These observations, together with the recognition that cell division, telomerase activity, telomeric shortening, and cell death occur in myocytes with aging and cardiac failure, suggest that adult heart has the ability to regenerate at least to a certain extent. Probably, this is because a pool of primitive cells reside in the myocardium and can form myocytes, smooth muscle cells (SMCs), and endothelial cells (ECs). This finding provides a different perspective concerning the biology of the heart and the mechanisms of myocardial homeostasis and tissue repair (Anversa, Kajstura et al. 2006) (Figure 21).

2.4 Metabolism of cardiomyocytes

Under non-ischemic conditions almost all (~95%) of ATP formation in the heart comes from oxidative phosphorylation in the mitochondria, with the remainder derived from glycolysis and GTP formation in the citric acid cycle. The heart has a relatively low ATP content (5μmol/g wet wt) and high rate of ATP hydrolysis (~0.5μmol g wet wt^{-1} s^{-1} at rest), thus there is complete turnover of the myocardial ATP pool approximately every 10 s under normal conditions (Ingwall 2001; Opie 1991). Approximately 60–70% of ATP hydrolysis fuels contractile shortening, and the remaining 30–40% is primarily used for the sarcoplasmic reticulum $Ca2^{+}$-ATPase and other ion pumps (Gibbs, 1978; Suga, 1990). In the well-perfused heart, ~60–90% of the acetyl- CoA comes from β-oxidation of fatty acids, and 10–40% comes from the oxidation of pyruvate that is derived in approximately equal amounts from glycolysis and lactate oxidation (Stanley et al., 2005).

The main substrate is Fatty Acid and cardiomyocytes also can use glucose, lactate and ketone. Glycolytic substrate is derived from exogenous glucose and glycogen stores. Studies assessing the effect of inhibition of glycolysis suggest that glycolytically generated ATP is preferentially used by the sarcoplasmic reticulum to fuel $Ca2^{+}$ uptake and by the sarcolemma to maintain ion homeostasis. Plasma ketone bodies are formed from fatty acids in the liver, and the arterial plasma concentration is normally very low, thus they are normally a minor substrate for the myocardium and could be used on pathological conditions (Stanley et al., 2005) (Figure 22).

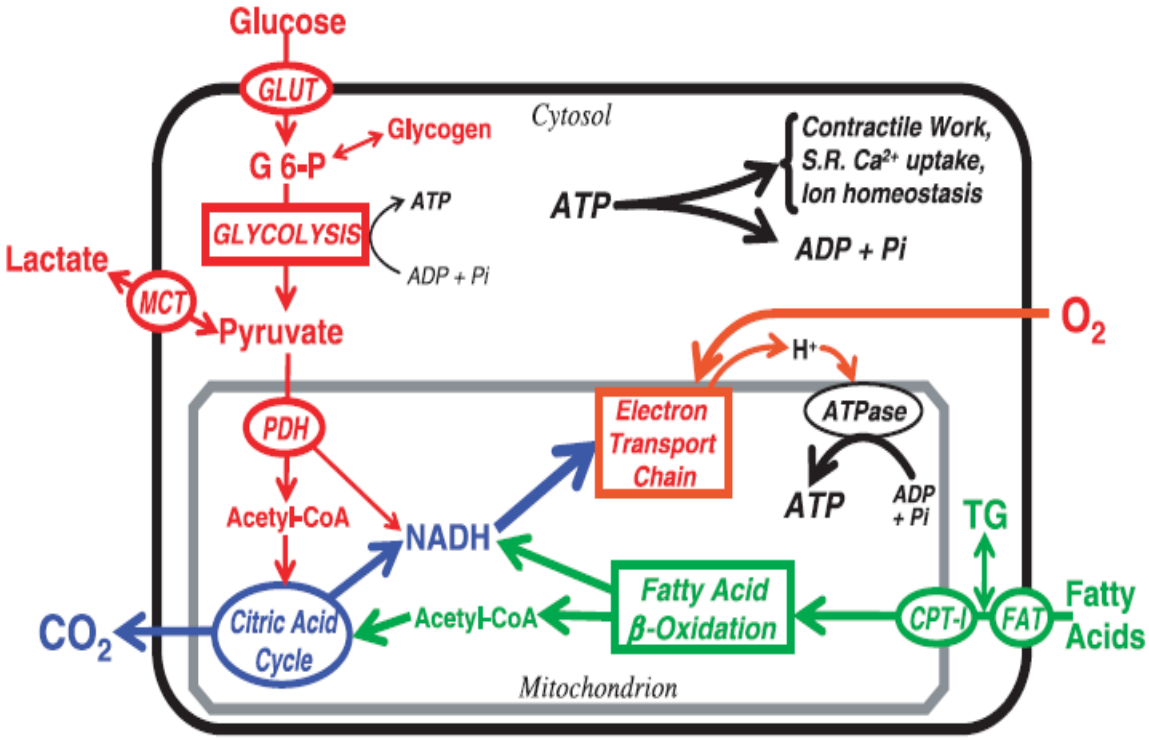


Figure 22. The pathways and regulatory points of myocardial substrate metabolism. CPT-I, carnitine palmitoyltransferase-I; FAT, fatty acid transporter/CD36; G 6-P, glucose 6-phosphate; GLUT, glucose transporters; MCT, monocarboxylic acid transporters; PDH, pyruvate dehydrogenase (Stanley et al., 2005).

3. Apoptosis signaling and the heart

Apoptosis signaling plays an important role in heart development. Cell death implying apoptotic-like DNA damage occurs during embryonic heart development (Takeda, Yu et al. 1996; Watanabe, Choudhry et al. 1998; Ya, van den Hoff et al. 1998). Experiments using Caspase inhibitors suggest that Caspase-dependent cell death occurs in the cardiac regions submitted to important morphological changes, such as the ventriculoarterial connections, the atrioventricular cushions, and the conduction tissue (Watanabe, Jafri et al. 2001; Cheng, Wessels et al. 2002; Schaefer, Doughman et al. 2004). Therefore, Caspase activation is likely assuring the proper formation of the cardiac cavities and the correct docking of the arteries to the ventricles during development.

Null mutation of some components of the death receptor pathway, such as Fas-associated death domain (FADD), Caspase-8 and cFLIP induce ventricular cell hypoplasia and non compaction of the ventricular wall in the embryo (Varfolomeev, Schuchmann et al. 1998; Yeh, Pompa et al. 1998; Yeh, Itie et al. 2000). Interestingly, the role of these proteins in heart development seems to be independent of cell death because deficiency in the pro-apoptotic factors FADD and Caspase-8, as well as lack of anti-apoptotic cFLIP, result in the same cardiac phenotype. All these mutations induced ventricular cell hypoplasia and non compaction of the ventricular wall in the embryo (Varfolomeev, Schuchmann et al. 1998; Yeh, Pompa et al. 1998; Yeh, Itie et al. 2000). Therefore, although cardiomyocyte-specific and/or inducible gene deletion would be required to confirm the specificity of the above mentioned cardiac phenotypes, genetic and biochemical evidences suggest that the extrinsic pathway regulates cardiomyocyte differentiation and cardiac response to stress through cell death-independent mechanisms that remain to be explored. On the contrary, experimental overexpression of Fas or FasL induces hypertrophy in cardiomyocytes (Nelson, Setser et al. 2000; Wollert, Heineke et al. 2000; Badorff, Ruetten et al. 2002). Cardiac hypertrophy ensues also from TNF-α stimulation *in vitro* (Yokoyama, Nakano et al. 1997; Condorelli, Morisco et al. 2002) and *in vivo* (Dibbs, Diwan et al. 2003; Janczewski, Kadokami et al. 2003). In addition, mice lacking TNF receptors 1 and 2 have larger infarct areas than wild-type animals (Kurrelmeyer, Michael et al. 2000), and TNF-α genetic deficiency decreases the hypertrophic growth of the myocardium after aortic banding (Sun, Chen et al. 2007). These data suggest that cardiac death receptor activation could play a relevant role in cardiomyocytes differentiation and growth.

It is now evident that apoptosis plays a key role in the pathogenesis of a variety of cardiovascular diseases. Many studies have reported that apoptosis occurs in myocardial tissue samples from patients suffering from myocardial infarction, dilated cardiomyopathy and end-stage heart failure (Narula, Haider et al. 1996; Olivetti, Quaini et al. 1996; Saraste, Pulkki et al. 1997; Aharinejad, Andrukhova et al. 2008) as well as in animal models of ischemia–reperfusion injury (Gottlieb, Burleson et al. 1994; Cheng, Kajstura et al. 1996; Gao, Yin et al. 2008).

However, increasing experimental evidence suggests that Caspase activation is not relevant for post-mitotic cardiomyocyte cell death (Ohno, Takemura et al. 1998;

Knaapen, Davies et al. 2001; Chen, Won et al. 2002; Kubasiak, Hernandez et al. 2002; Bahi, Zhang et al. 2006). Our lab and others reported that most of the apoptotic regulators are repressed during development (Bahi, Zhang et al. 2006; Madden, Donovan et al. 2007). The silence of the apoptotic regulators could be critical for the apoptosis. Our previous data have shown that the level of Apaf-1 expression is important for the induction of apoptosis in cardiomyocytes (Sanchis et al., 2003).

In addition, controversy also exists as to the mechanisms conferring cardio-protection by Caspase inhibitors, which could be unrelated to apoptosis or target non-myocardial cells (Yaoita, Ogawa et al. 1998; Okamura, Miura et al. 2000; Ruetten, Badorff et al. 2001; Hayakawa, Takemura et al. 2003). It has been demonstrated that cardiac graft salvage in lpr (Fas loss-of-function mutation) and gld (Fas ligand loss-of-fuction mutation) mice is due to a general impairment of the host immune response, which normally would attack the graft, and not to a specific role of cardiac Fas signaling (Djamali and Odorico, 1998). During ischemia/reoxygenation-induced cardiomyocyte death, Fas and FasL have been shown recently to promote granulation tissue death but not cardiomyocyte loss (Li, Takemura et al. 2004; Gomez, Chavanis et al. 2005). These results agree with the finding that the main role of Caspase inhibitors given during experimental myocardial infarction is to protect non-myocardial cells (Hayakawa, Takemura et al. 2003). TNF-α genetic deficiency reduced cardiac inflammation and fibrosis, suggesting a role of TNF-α in granulation tissue signaling (Sun, Chen et al. 2007). Myocyte DNA fragmentation and Caspase activation (TUNEL signal) was inhibited by Caspase inhibitors without reduction of the infarct size in ischemia–reperfused rat hearts using YVAD (Caspase-1 inhibitor) or DEVD (Caspase-3 inhibitor) (Okamura, Miura et al. 2000) or attenuating the I/R injury using zVAD (pan-Caspase inhibitor) (Yaoita et al., 1998) or aurintricarboxylic acid (ATA), an endonuclease inhibitor (Zhao, Morris et al. 2003). But Knaapen et al. Showed that in heart failure TUNEL-positive cardiomyocytes were negative for active Caspase-3 (Knaapen et al., 2001). In I/R heart TUNEL staining was marked, but ssDNA and cleaved Caspase-3 staining were significantly less (P<0.001 compared with TUNEL), and Caspase-3 and Caspase-8 activities per milligram protein were not significantly different from those in normal hearts (French et al., 2009). Langendorff heart I/R model showed

that ischemia/reperfusion initiates a cell death pathway that is independent of Caspases but requires calpain and mitochondrial dysfunction (Chen, Won et al. 2002). The sodium/hydrogen exchanger inhibitor prevented calpain activation after I/R. Calpain inhibitors prevented cleavage of Bid as well as the downstream indices of cell death, including DNA strand breaks, creatine kinase (CK) release, and infarction measured by triphenyl tetrazolium chloride (TTC) staining. In contrast, the broad spectrum Caspase inhibitor IDN6734 was not protective in this model. The phenotype of cyclophilinD null mice suggested the importance of mitochondria in cardiac cell damage (Baines, Kaiser et al. 2005). Wild-type and Ppif (encoding cyclophilinD)-null mice were also subjected to cardiac ischemia/reperfusion injury, and a 40% reduction in infarction area was observed in the Ppif null mice, nearly identical to the level of protection observed using CsA in wild-type mice. Another thing we should keep in mind is that some of investigations are from cell lines (Ekhterae, Lin et al. 1999; Neuss, Monticone et al. 2001).

Therefore an insight into apoptotic gene expression during development and its regulation could contribute to improve our knowledge of the relationship of apoptosis and heart development and disease. In our opinion, if Caspase-dependent apoptotic pathway is by-passed developmentally an alternative way controlling post-mitotic cardiomyocytes cell death is worth to be explored.

Hypothesis And Objectives

Hypothesis:

Apoptotic gene expression is silenced during development in the heart inducing a switch in the mechanisms involved in pathological cardiomyocyte death.

Main objectives:

1. To characterize the regulation of the expression of apoptotic genes in cardiomyocytes during heart development.
2. To identify alternative pathways involved in cardiomyocyte cell damage.

Experimental objectives:

a. To identify the apoptotic genes silenced during heart development and to assess the potential re-expression of these genes during cell stress.
b. To identify the pathways that regulate ischemia induced DNA damage and how these processes are controlled after apoptotic genes, including caspases, are silenced in postmitotic cardiomyocytes.
c. To identify the pathways controlling apoptotic gene expression during heart development.

Results

Results

Block 1.

Developmental silencing and independency from E2F of apoptotic gene expression in postmitotic tissues.

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Developmental silencing and independency from E2F of apoptotic gene expression in postmitotic tissues

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Abstract The involvement of caspases in postmitotic cell death is controversial. Here we report that adult brain and heart are devoid of many key pro-apoptotic proteins due to a progressive postnatal silencing event involving a reduction of their transcript levels. E2F has been shown to control cell cycle progression and to be transcriptional activator of apoptotic genes. However, our data demonstrate that apoptotic gene expression in heart, brain and liver, as well as cardiac and neuronal apoptotic gene silencing during development, are E2F-independent events. Therefore, the genes regulating caspase-dependent cell death are expressed in embryonic organs in an E2F-independent manner and a developmental-related silencing event represses these genes in postmitotic adult tissues.

Keywords: Heart; Brain; Development; Apoptosis; Caspase; E2F

1. Introduction

Deregulation of the caspase-dependent cell death process has been involved in the onset and progression of autoimmune diseases [1,2], and cancer [3,4]. However, despite a considerable effort directed to untangling the processes implicated in cardiac and neuronal diseases involving cell loss, the relevance of caspases in these diseases remains debated. Many reports of cytochrome c translocation, nuclear condensation and chromatin DNA degradation during cardiac and neuronal disease suggest the involvement of caspases in these situations. However, other reports have challenged the relevance of caspase activation in cardiomyocyte and neuron death in these pathologies [5–9]. Changes in the expression of key regulators of the cell death pathway related to the experimental models used, which include from cell lines to adult animals, could underlie the differences in the results.

We previously reported that although embryonic cardiomyocytes express the proteins regulating caspase activation, low levels of these proteins are found in the adult heart, concomitant with a switch to caspase-independent mechanisms being involved in ischemia-induced cardiomyocyte damage [9]. Others have reported low levels of Apaf-1 in cortical neurons [10], retinal neurons [7] and sympathetic neurons [11], as well as low levels of caspase-3 and pro-apoptotic Bcl-2 family members in retinal neurons [7,8]. However, some reports have shown high expression of caspases 3 and 9 in neonatal neurons [11], sustained caspase-9 expression in brain [10,12,13] and high expression of Apaf-1 and some caspases in the heart [13–15]. Therefore, controversy exists about the extent and magnitude of the changes on apoptotic gene expression in postmitotic cells.

Our aim was to characterize the timing and broadness of the silencing event affecting the genes regulating the caspase-dependent death pathway during heart and brain development, and to get some clues into the underlying mechanisms. We show that the development-related silencing of the main genes involved in the control of caspase-dependent cell death affect the heart and the brain, and to a lesser extent the liver, in both rat and mouse. In general, this event initiates after birth in rodents and culminates during adulthood, with some genes being expressed only in embryos, such as Caspase-2, and others, like Bcl-XL, expressed steadily until adulthood. The apoptotic genes are not re-expressed in vitro in adult cardiomyocytes during stress situations. Our results also show that embryonic expression of the caspase-dependent cell death machinery and its silencing is not influenced by the transcription factor E2F1, despite the fact that E2F1 controls the expression of some of these genes in other cell types.

2. Materials and methods

2.1. Animals, tissues and cells

Sprague–Dawley rats were housed in our Experimental Animal Service under standard conditions. Wild-type and mice deficient for E2F1, E2F2 or both genes were housed and kept at the Animal Facility, University of the Basque Country, as previously reported [16]. Animals were sacrificed in compliance with the guidelines defined by our Institutional Ethical Committee, which approved the experimental design of this project. Heart, brain and liver were dissected, minced into small cubes, rinsed with cold phosphate buffered saline and snap-frozen into liquid nitrogen. Embryonic and adult cardiomyocytes were prepared and stored as described elsewhere [9]. Other primary cells, tissues and cell lines were a kind gift from members of our lab.

2.2. Adult ventricular myocyte culture and lentiviral infection

Adult ventricular myocytes were obtained from 9-month-old male Sprague–Dawley rats and cultured as previously described [9]. Plates were either treated with 1 or 10 nM Angiotensin-II (SIGMA) for

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48 h in standard medium as described by others [17] or submitted to 6 h of ischemia (Tyrode's solution, 0.1% O_2 in an InVivo400 hypoxic chamber, see details in [9]) followed by 18 h of reoxygenation in standard medium, as described by Kang et al. [18], or left untreated. For inducing E2F1 expression, the fragment HindIII–EcoRI of the vector pRc/CMV-HA-E2F1 (kind gift of Erik K Flemington, Dana-Farber Cancer Institute, Boston, USA), containing the open reading frame of human E2F1 followed by a hemaglutinin tag (HA) was subcloned into the PmeI site of the lentiviral overexpression vector pWPI, then lentivirus particles carrying the empty vector or the E2F1 expression vector were obtained and added to the cell cultures as described previously [9] for 72 h. E2F1 expression was monitored by immunodetection of the HA tag.

2.3. Detection of protein expression

Protein expression was detected in protein extracts diluted in Tris-buffered 2% SDS solution at pH 6.8 as previously reported [9]. Antibody data can be found in the Supplementary Table 1. Each immunodetection was repeated with two to three different membranes containing samples coming from different animals of each age.

2.4. Analysis of the steady-state transcript levels

Total RNA was obtained from frozen tissues with the RNA Spin Mini kit (GE Healthcare). RNA concentration measurements and retrotranscription were done as described [9]. Equal volumes of cDNA were amplified by PCR using a couple of specific primers expanding at least two exons within the gene of interest (see Supplementary Table 2 for details) as described elsewhere [9]. Ten microlitre aliquots were taken at PCR cycles 25, 30 and 35, mixed with SYBR Safe dye (Invitrogen) and migrated in a 1% agarose gel. Densitometry of the DNA bands was performed with the Scion Image software (Scion Corporation) and qualitative calculations were done comparing data from non-saturated products (usually, corresponding to PCR cycle 30). Loading was checked by amplification of the unr transcript [9]. Transcript analysis was performed from three samples of each tissue coming from different animals.

3. Results

3.1. Expression of the genes implicated in the caspase-dependent signalling is silenced during postnatal development, notably in heart and brain

Expression of proteins involved in the control of caspase-dependent cell death was analyzed in tissues from rats of different ages ranging from embryos to adults by using the antibodies described in Supplementary Table 1. Antibodies that have been checked against knock out mice or knocked down cultured cells were used when possible. Our results show that the genes involved in the induction of cell death were intensely repressed in the adult heart and, most of them, also in the brain. Exceptions were caspase-2, which was only expressed in embryos, and Bak and Bax, which were downregulated in all tissues during postnatal development (Fig. 1 and Supplementary Fig. 1). The expression of caspase-9 and executioner caspases 3 and 7 was reduced in postnatal heart and brain. We checked the specificity of caspase-9 antibodies supplied by Alexis and Cell Signaling comparing the signal obtained in the same blot for human and rodent cells (Supplementary Fig. 2). We concluded that caspase-9 expression is reduced in adult heart and brain when compared to the embryonic stage. With regard to the expression of anti-apoptotic proteins, Bcl-2 was silenced in heart and brain during development and undetectable in the liver, whereas Bcl-XL was expressed in all tissues until adulthood (Fig. 1).

Decreased expression of the proteins involved in caspase-dependent cell death has been frequently associated with post-translational modifications, involving a reduction of protein stability [19]. To ascertain whether the developmental silencing process affected the steady-state amount of the corresponding transcripts, we performed semi-quantitative RT-PCR in RNA extracts from embryo and adult tissues, as described in Section 2. Our results demonstrated that expression silencing affects the amount of mRNA pointing to a decrease in transcription (Fig. 2).

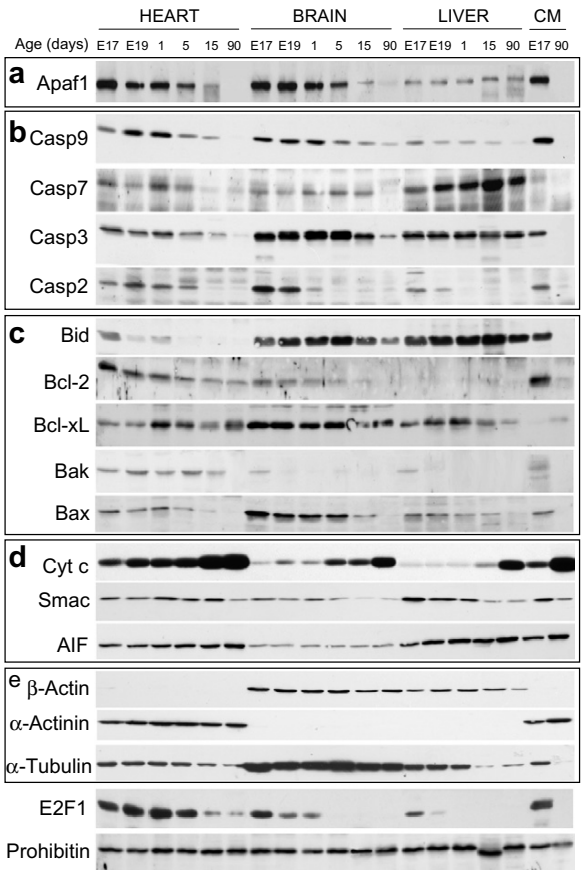

Fig. 1. Detection of proteins involved in apoptosis at different developmental stages in heart, brain and liver. Protein extracts from cardiac ventricles, brain homogenate and liver were collected from rats at ages ranging from embryonic day 17 (E17) to 3-month-old adults. Protein detection was carried out with the antibodies listed in the Supplementary Table 1. The image is representative of the results obtained with samples coming from three different animals per age. a: Adaptor proteins; b: caspases; c: Bcl-2 family proteins; d: mitochondrial proteins (Cyt *c*: cytochrome *c*); and e: structural proteins. CM: primary isolated rat cardiomyocytes.

3.2. E2F1 is neither involved in apoptotic gene expression in the embryo nor does its development-linked repression affect apoptotic gene expression in adult tissues

E2F transcription factors are well known by their role in the control of cell cycle progression. Recently, E2F1 has been suggested to be transcriptional activator of some genes regulating the caspase activation cascade, such as Apaf-1 [20–23], caspase-8 [24,25], caspase-3 [23,25], caspase-7 [22,23,25], Bid [24] and Smac [26]. Our results showed that E2F1 expression decreases during differentiation in all tissues analyzed and its

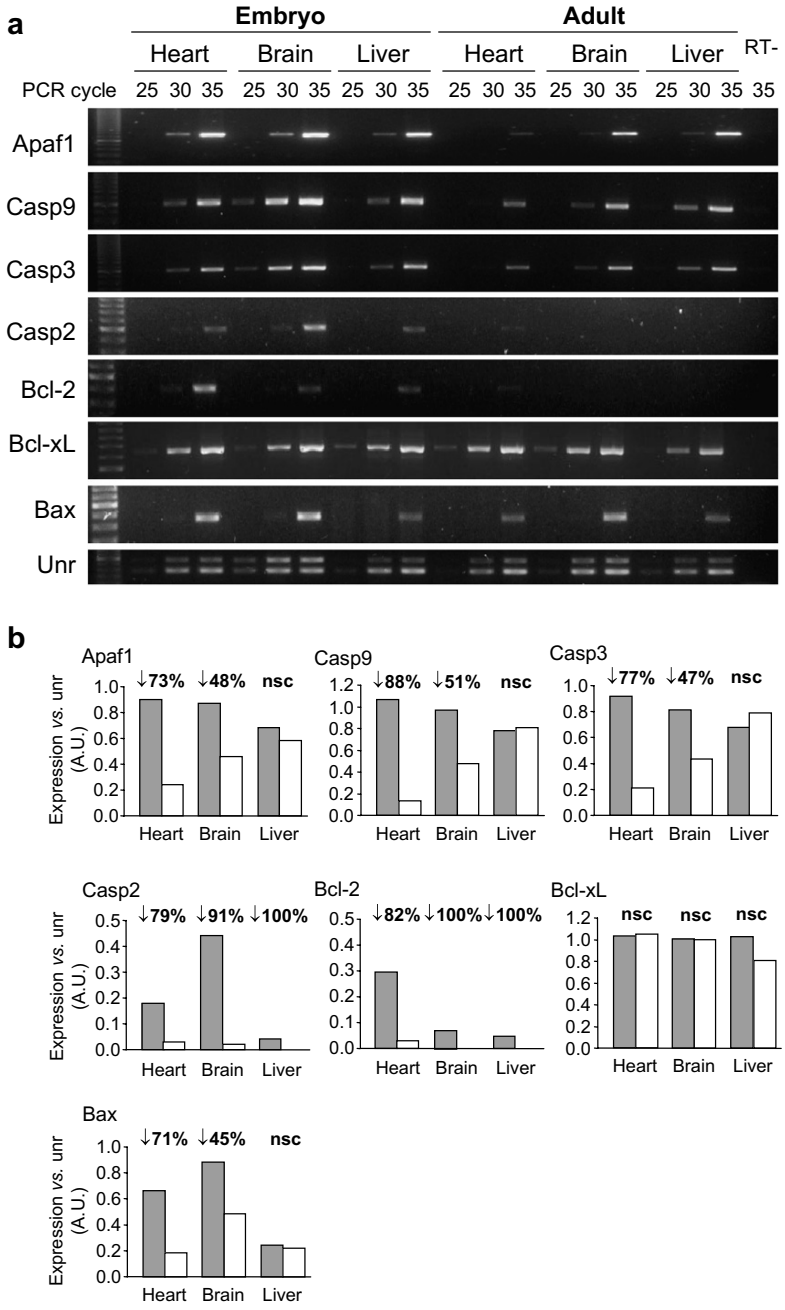

Fig. 2. Analysis of the transcript levels for different genes involved in apoptosis, in embryonic and adult heart, brain and liver. (a) Total RNA was extracted from cardiac ventricles, brain homogenate and liver of E17 embryos and 3-month-old rats. RNA was retrotranscribed and the expression of the genes of interest was measured by PCR as described in Section 2. RT-: PCR product at cycle 35 from a non-retrotranscribed neonatal heart RNA sample. (b) Bar graphs represent the semi-quantification of each specific gene in arbitrary units (AU), corrected by loading (upstream of n-Ras gene expression); gray bars: E17 embryo; white bars: 3-month-old adult. For each gene, differences between embryo and adult expression are shown as percentage. Values are percent expression change between embryo and adult. nsc: no significant change. Graphs are representative of three experiments made with independent tissue samples.

expression level is proportional to the expression levels of many apoptotic genes in the embryo, pointing to a role of E2F in the control of the genes regulating caspase-dependent death in heart, brain and liver (Fig. 1). However, E2F1 and E2F2 single and double knockout mice showed no differences neither in the embryonic expression of these genes nor in their repression during development when compared with their wild-type littermates (Fig. 3). These data demonstrated that, although E2F factors can act as transcriptional activators of key genes regulating the caspase signalling cascade in embryonic and tumor cells, they do not control the normal expression of these genes in heart, brain and liver.

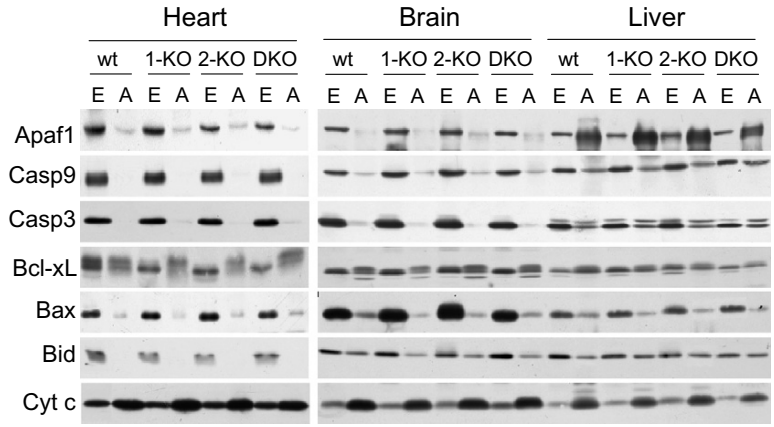

Fig. 3. Expression of proteins involved in apoptosis in the heart, brain and liver of embryos and adult mice from wild-type and E2F1, E2F2 or double E2F1-2 knock out strains. Total protein extracts were obtained as described in Section 2 and protein expression was detected by Western blot using the antibodies listed in the Supplementary Table 1. Images are representative of three independent experiments with comparable results. wt, wild-type; KO, E2F knockout; DKO, E2F1-2 double knockout. E: E19 embryo; A: P60 adult.

3.3. The main regulators of the caspase-dependent cell death are not re-expressed in cultured adult cardiomyocytes submitted to stress stimuli

In order to investigate whether the apoptotic machinery can be re-expressed after a stress input, the expression of apoptotic genes was analyzed in primary adult ventricular myocytes cultured for 48 h with 1 or 10 nM Angiotensin-II, which has been reported to induce hypertrophy as well as apoptotic-like DNA damage and cell death [17], or in cardiomyocytes after 6 h of ischemia and 18 h of reoxygenation, which induces cell death, cytochrome c release and apoptotic-like DNA degradation [18]. The major regulators of the caspase-dependent death pathway were not re-expressed during these treatments (Fig. 4). Enhanced E2F1 expression can activate the expression of genes involved in apoptosis in embryonic fibroblasts and cell lines [25]. In order to verify whether E2F1 has similar effects in adult ventricular myocytes, expression of apoptotic genes was analyzed three days after E2F1 transduction in these cells. Despite the increase in E2F1 expression, cardiomyocytes did not re-express any of the apoptotic genes (Fig. 4). Taken together, our results suggest that adult cardiac myocytes loss the ability to express the genes that regulate the caspase-dependent death pathway and, therefore, cytochrome c release, DNA damage and phosphatidylserine externalization [17,18] are likely caspase-independent events in these cells.

4. Discussion

Here we report that the proteins controlling caspase-dependent cell death are expressed in the embryonic heart, brain and liver, but are later silenced during postnatal development, especially in the heart and the brain. Of note, the expression of many genes controlling intermediate steps leading to caspase activation is always low in the liver, whose sensitivity to caspase-dependent cell death could depend on the high expression of Bid and executioner caspases 3 and 7. The gene silencing event implies a reduction in the amount of the transcripts coding for these proteins. Our analysis also demonstrates that E2F factors are not involved in the expression of the major regulators of the caspase signalling cascade in the embryo heart, brain and liver. Finally, the results presented here suggest that postmitotic cardiomyocytes do not re-express the

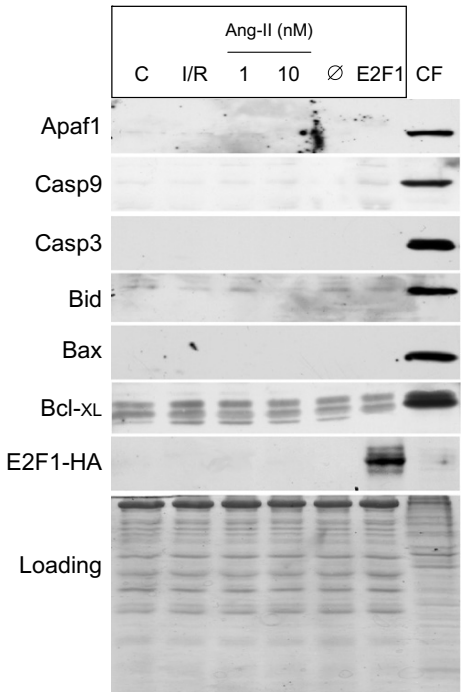

Fig. 4. The expression of the major apoptotic regulators is not enhanced in adult cardiomyocytes submitted to different stress stimuli reported to induce cell damage. Rat adult ventricular myocytes were treated with 1 or 10 nM Angiotensin-II (Ang-II) for 48 h, 6 h of ischemia and 18 h of reoxygenation (I/R), or were induced to overexpress E2F1 for 72 h (see Section 2). The expression of several genes involved in the caspase-dependent cell death pathway was immunodetected after SDS-PAGE in total protein extracts from control cardiomyocyes (C), cardiomyocytes transduced with empty vector (∅) and stressed cardiomyocytes. Control neonatal cardiac fibroblasts (CF). The image is representative of three independent experiments.

apoptotic genes neither during stress nor after an increase in E2F1 expression.

Although previous reports have shown that the expression of some apoptotic proteins is reduced in postnatal animals [7–15], simultaneous data argue in favour of high expression of some of these proteins [10–15]. From our point of view, clarifying this fact has important consequences for the correct understanding of the mechanisms engaged in cardiomyocyte and neuron cell death in the adult. Indeed, outstanding research on neurodegenerative and cardiac diseases is being frequently conducted or complemented with studies performed with embryonic cells and immortalized cell lines, which express the full caspase-dependent death machinery. Using these models, caspase activation has been proposed to play a relevant role in cardiomyocyte and neuron death during disease [27–29]. However, although the role of caspase-dependent events on the morphogenesis of the brain and the heart are well established [30–32], the role of caspases in postmitotic cell death is controversial [5–7,33].

Our results show that the pro- death genes in the caspase-dependent pathway are repressed at both protein and transcript level during development, in apparent contradiction with previous reports [14,15]. Although reduction in Apaf-1 expression and relatively unchanged levels of caspase-9 are observed when comparing embryos with early neonatal cardiomyocytes [15], in the present work we show that adult fully differentiated cardiomyocytes lack both proteins. A previous work showed that caspase-9 expression increases during heart development [14]. However, the size of the caspase-9 band shown in that report was smaller than expected and varied in size when comparing heart and brain extracts. We checked the specificity of two commercial antibodies against caspase-9. We found that only one antibody detected correctly the different size of the human and rodent caspase-9 isoforms. This antibody detected caspase-9 only in embryonic and at lesser extent in young heart, but not in adult heart and adult isolated cardiomyocytes. Therefore, we conclude that caspase-9 is actually silenced during development in heart and brain, alike the rest of caspases analyzed. Caspase-9 has been suggested to be expressed in adult mouse heart both at the protein and transcript levels [13]. In support of the decrease in caspase-9 expression in the myocardium, we report here that isolated adult cardiomyocytes are virtually devoid of caspase-9 (Figs. 1 and 4), suggesting that the remnant caspase-9 signal in adult heart comes from non-muscular cells. In addition, we have taken into account only non-saturated signals for our comparative transcript analysis (Fig. 2), which confirm an important decrease in caspase-9 transcript both in the heart and the brain.

If the key regulators of the caspase-dependent cell death pathway are silenced in postmitotic tissues, the suggested involvement of caspases in cardiac and neuronal diseases in the adult (e.g. [28,29,34]) could depend on the re-expression of the apoptotic signalling cascade in certain pathological conditions. The results presented here suggest that cultured primary adult cardiomyocytes can not re-express the apoptotic genes in vitro when submitted to certain death stimuli, although this does not rule out the re-expression of apoptotic genes in vivo. In neurons, expression of Apaf-1 has been reported to be enhanced in a model of brain injury [10] and Caspase-2 up-regulation occurs in certain neurons of a Huntington's disease mouse model [29]. However, experiments conducted to identify the mechanisms involved in neuronal and cardiac cell death during disease have been performed usually in embryonic cells. Our results put forward a note of caution on the interpretation of results obtained with embryonic cells or cell lines. We suggest the need to prove the implication of caspases in adult cells to confirm the apoptotic gene re-expression event, which would have important clinical consequences.

Our results also show that expression of apoptosis inducing factor (AIF), a potentially deleterious protein, is increased in the adult heart. Interestingly, we and others have demonstrated that AIF plays actually a beneficial role during reoxygenation in cardiomyocytes [35,9], and protects the heart and the brain against oxidative damage [35,36]. Another interesting fact is that the expression of pro-death proteins Bax and Bak, thought to play redundant roles, vary when comparing different tissues. While both Bax and Bak are expressed in the young heart, Bak is the major Bcl-2-related pro-death member in the brain, whereas Bax is more expressed than Bak in the liver. These results suggest that the relevance of Bak and Bax in the control of the mitochondrial events leading to cytochrome c release is tissue-specific. Finally, our results show that Bcl-XL expression is sustained at significant levels until adulthood, suggesting that it could be a crucial protector of mitochondrial integrity in the adult brain and heart.

Regarding the mechanisms involved in apoptotic gene down-regulation, our data discard an essential role of E2F1, despite the fact that this protein has been involved in the expression of key regulators of the caspase-dependent pathway in proliferating cells and in the immune system [20–26,37–40]. The lack of effects on the expression of these genes in E2F1 and 2 double null mice is unlikely due to compensation by other members of the E2F1 family because E2F1 has been described to be the only member able of inducing apoptotic gene expression [39], and because the ability of other E2F proteins for inducing apoptosis rely on endogenous E2F1 expression [40].

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2007.11.046.

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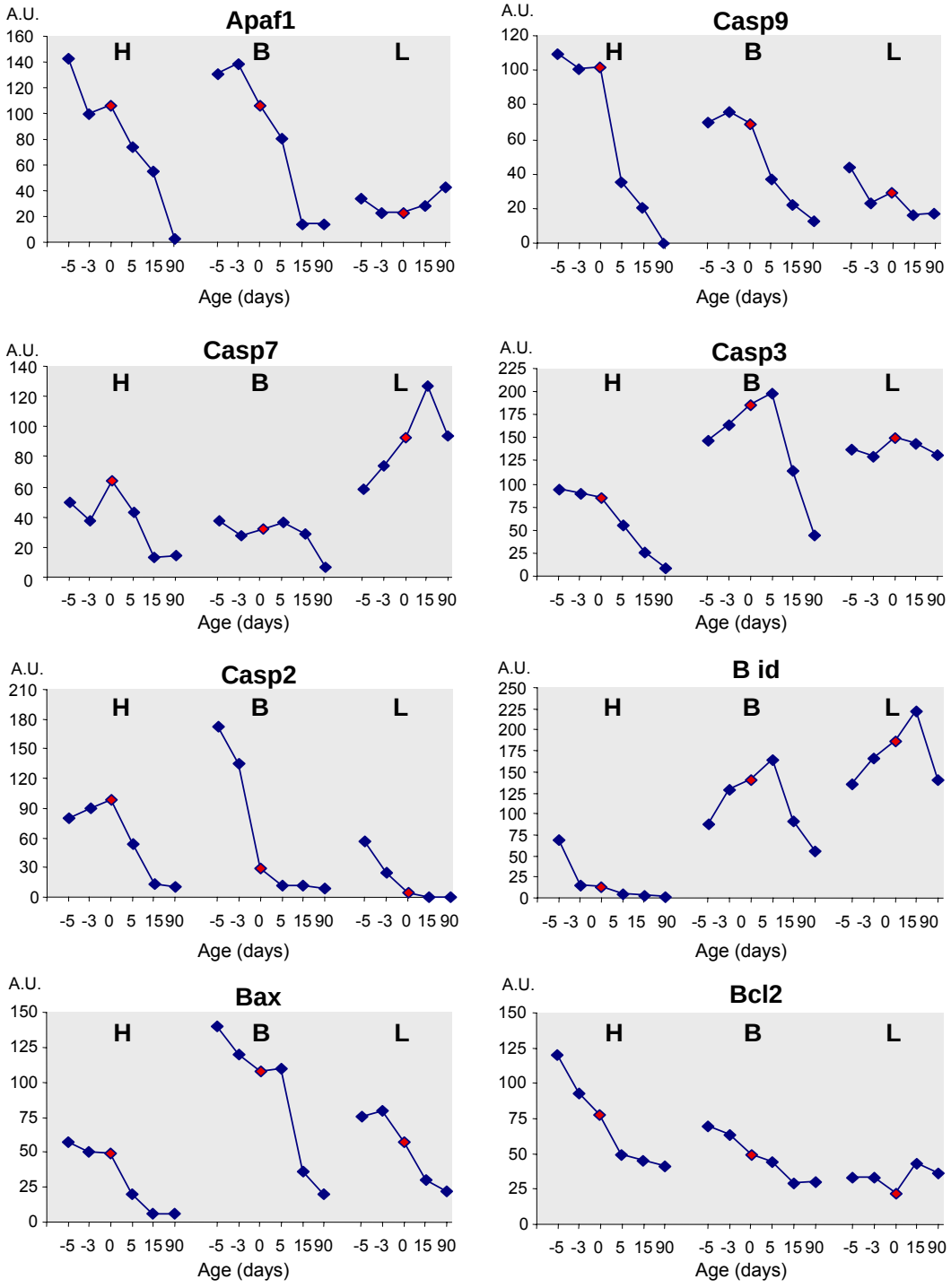
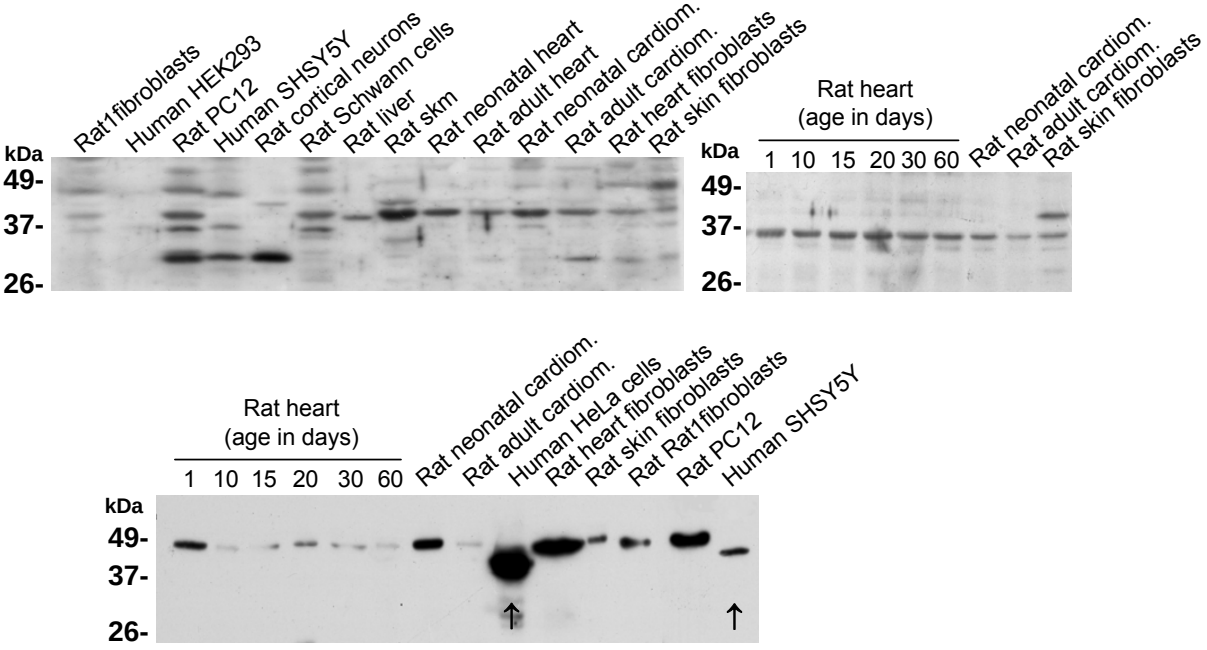
Supplementary Figure 1. **Western Blot densitometry data from the analysis of apoptotic gene expression in Heart, Brain and Liver during development.** Densitometry values corresponding to the experiments reported in Figure 1 were obtained using the Scion Image software (Scion Corporation). Values are expressed as arbitrary units (A.U.). Red rhomb signals birth. Data are representative of three independent experiments.



H: Heart; B: Brain; L: Liver. A.U.: Arbitrary units. ◆ : Birth

Supplementary Figure 2. **Specificity analysis of caspase-9 antibodies.** Protein extracts from several tissues and cells were analyzed for the expression of caspase-9 with two antibodies reported to detect endogenous expression of the protein in human and rodent samples. Upper panels: Alexis ALX-210-015 polyclonal rabbit antibody. Lower panel: Cell Signaling 9508 monoclonal mouse antibody. Arrows indicate the smaller size of the α-caspase-9 human isoform (46 kDa) compared to the rat protein (50 kDa). Abbreviations: skm, hind leg skeletal muscle; cardiom., primary cardiomyocytes.



Results shown in Suppl. Figure 2:
Although the major isoform of caspase-9 has a molecular weight of 50 and 46 kDa (in rodents and humans, respectively), the Alexis antibody detected unspecific bands smaller than 45 kDa in rodent samples (PC12 naive neuroblastoma cells, Rat1 fibroblast cell line, and rat tissues) and human cell lines (HEK293 and SHSY5Y neuroblastoma cells) (Suppl. Figure 2, left upper panel). The protein detected by this antibody was uniformly expressed in rodent heart through postnatal life (Suppl. Figure 2, upper right panel). On the contrary, the caspase-9 Cell Signaling antibody correctly discriminated the size of endogenous caspase-9 isoform alpha from rodent and human samples (Suppl. Figure 2, lower panel, right). This antibody detected a marked reduction in the expression of caspase-9 in rat heart and isolated cardiomyocytes during postnatal development (Suppl. Figure 2, lower panel, left). Therefore, we concluded that caspase-9 expression is reduced in adult heart and brain when compared to the embryonic stage.

Results

Block 2.

Switch from caspase-dependent to caspase-independent death during heart development: essential role of endonuclease G in ischemia-induced DNA processing of differentiated cardiomyocytes.

Bahi N, Zhang J, Llovera M, Ballester M, Comella JX, Sanchis D.

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Switch from Caspase-dependent to Caspase-independent Death during Heart Development

ESSENTIAL ROLE OF ENDONUCLEASE G IN ISCHEMIA-INDUCED DNA PROCESSING OF DIFFERENTIATED CARDIOMYOCYTES[*][S]

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Differentiated cardiomyocytes are resistant to caspase-dependent cell death; however, the mechanisms involved are still uncertain. We previously reported that low Apaf1 expression partially accounts for cardiomyocyte resistance to apoptosis. Here, we extend the knowledge on the molecular basis of cardiac resistance to caspase activation by showing that the whole caspase-dependent pathway is silenced during heart development. Experimental ischemia triggers caspase activation in embryonic cardiomyocytes and proliferating fibroblasts, but not in neonatal and adult cardiomyocytes. Ischemia induces the release of the proapoptotic factors cytochrome *c*, truncated-AIF, and EndoG from mitochondria in postnatal cardiomyocytes in the absence of caspase activation. On the one hand, lentiviral-driven knockdown of EndoG shows that this gene is essential for ischemia-induced DNA degradation in neonatal cardiomyocytes, but not in proliferating fibroblasts; on the other hand, the AIF gene is essential for high molecular DNA cleavage in fibroblasts, but not in postmitotic cardiomyocytes, where it plays a prosurvival role during reoxygenation. These results show the switch from caspase-dependent to caspase-independent death pathways after cardiac cell differentiation, and disclose the relevance of EndoG in the caspase-independent DNA processing of differentiated cardiomyocytes.

Apoptosis, the best characterized type of programmed cell death (PCD),[6] is executed by a family of cysteinyl aspartate proteinases known as caspases (1). Activation of caspases, either by extrinsic or intrinsic signals, is regulated by a set of proteins, including the components of the death-inducing signaling complex (DISC), in the death receptor-mediated extrinsic pathway, and the Bcl-2 family of anti- and proapoptotic proteins, as well as the apoptosome complex including Apaf-1, in the intrinsic pathway, which involves the mitochondrion (2). The apoptotic process ends with the degradation of nuclear DNA by the caspase-dependent endonuclease CAD/DFF40 (3).

Although the order in which every step of caspase-dependent PCD takes place is well defined in proliferating and undifferentiated cells, the precise cell death pathways activated in differentiated cardiomyocytes and neurons remain more elusive (4–6). In cardiomyocytes, the prevailing dogma that the caspase-dependent machinery is fully functional has been recently challenged (4, 7). In addition, although cardiac apoptotic rates of 0.1–0.01% occurring during cardiac disease have been estimated to be pathologically relevant (8, 9), the underlying mechanisms for the low incidence of apoptosis have not been addressed. Nevertheless, apoptotic cells have been detected in the heart and in cardiomyocyte cultures during ischemia (10–13). Therefore, cardiac ischemia is a clinically relevant model for analyzing the mechanisms that promote death in differentiated cardiomyocytes.

Potential executors of caspase-independent cell death have been discovered. The mitochondrial protein apoptosis-inducing factor (AIF) was identified as an important executor of cell death when translocated to the nucleus (14). Translocation of AIF has been proposed to be both caspase-dependent (15, 16) and caspase-independent (17–19), although the reason for this discrepancy is uncertain (20). Furthermore, the death-inducing role of AIF in differentiated cells is controversial. Indeed, the participation of AIF in cell death has been either confirmed (19, 21–23) or discarded (24, 25). Thus, the role of AIF in promoting cell death and the mechanisms involved in promoting its release from mitochondria need to be better characterized.

In the nematode *Caenorhabditis elegans*, AIF is a component of the caspase-dependent mitochondrial pathway, which also involves the mitochondrial endonuclease EndoG (26). However, the role of EndoG in apoptotic DNA damage was initially suggested to be caspase-independent (27, 28). Furthermore,

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[S] The on-line version of this article (available at http://www.jbc.org) contains supplemental Table S1 and Figs. S1–S3.

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[6] The abbreviations used are: PCD, programmed cell death; AIF, apoptosis-inducing factor; DPQ, 3,4-dihydro-5[4-(1-piperindinyl)butoxy]-1(2*H*)-isoquinoline; DIQ, 1,5-dihydroxyisoquinoline; EndoG, endonuclease G; PARP, poly-ADP-ribose polymerase; Z, benzyloxycarbonyl; fmk, fluoromethylketone; TNF, tumor necrosis factor; shRNAi, small hairpin RNA interference.

recent data from EndoG deficient mice do not apparently support a relevant role of this protein in mammalian cell death and DNA processing (29, 30). Therefore, the role of EndoG in mammalian cell death and the mechanisms involved in its release from mitochondria deserve further investigation.

While studying the mechanisms involved in cardiomyocyte cell death, we found that these cells down-regulate the expression of caspases after birth. Therefore, we checked the role of several known caspase-independent effectors of cell damage by using a lentiviral-driven gene knockdown approach. Results herein reported prove that cardiomyocytes lose the competence to dye by apoptosis after the caspase-dependent machinery is silenced during development, and show the existence of a caspase-independent, yet mitochondria-dependent, cell death pathway involving translocation and activation of EndoG, leading to the degradation of nuclear DNA, independently of AIF.

EXPERIMENTAL PROCEDURES

Chemicals—Executor caspase-specific substrate Z-Asp-Glu-Val-Asp-AFC (Z-DEVD-AFC), caspase-8-specific substrate Z-Ile-Glu-Thr-Asp-AFC (Z-IETD-AFC), pan-caspase inhibitor Z-Val-Ala-Asp(OMe)-CH_2F (z-VAD-fmk), poly-ADP-ribose polymerase (PARP) inhibitors 3,4-dihydro-5[4-(1-piperindinyl)butoxy]-1(2*H*)-isoquinoline (DPQ) and 1,5-dihydroxyisoquinoline (DIQ), and calpain inhibitor carbobenzoxy-valinyl-phenylalaninal (MDL 28170) were purchased from Calbiochem. Other chemicals were purchased from Sigma unless otherwise stated.

Cell Culture, Treatments, and Viability Measurements—Rat neonatal cardiomyocytes and heart fibroblasts were obtained from the heart of 3–5-day-old Sprague-Dawley rats as described elsewhere (4, 31). Embryonic cardiomyocytes (E16) were obtained following essentially the same protocol, but reducing the time of digestion to 15 min, and were cultured in the same medium enriched with 1 μM insulin (Sigma), 1 ng/ml cardiotrophin-1 (R&D Systems), and 10 nM mouse epidermal growth factor (Upstate). Adult cardiomyocytes (P180) were obtained and cultured following the protocol described by Ravassa *et al.* (32). Experimental ischemia was achieved by culturing cells in Tyrode's solution (NaCl 137 mM, KCl 2.7 mM, Na_2HPO_4 8 mM, KH_2PO_4 1.5 mM, $CaCl_2$ 0.9 mM, and 0.5 mM, initial pH: 7.2) inside a hypoxic chamber (Billups-Rothenberg) in a mixture of 5% CO_2 and 95% N_2 following manufacturer's instructions to attain a 0.1% oxygen concentration (31). Quantification of cell death was performed by the trypan blue exclusion assay at the end of treatments as reported (31). Cell death in each experimental condition was measured in duplicates, and error bars represent the S.E. of three independent experiments.

Enzymatic Caspase Activity Assay—Caspase-8 (Z-IETD-AFC) and executioner (Z-DEVD-AFC) caspase activity were measured in cell extracts as previously reported (4), using the fluorogenic substrates at 50 μM in 96-well plates. Fluorescence was detected with a Bio-tek FL 600 fluorometer (Izasa). Data obtained for different experimental conditions were compared within the linear phase of absorbance increase. Data are the mean of three independent experiments performed in duplicates.

Cytosolic Extracts—Cytosolic fractions were obtained as previously reported (4) at the end of the treatments to detect the release of apoptotic factors from the mitochondria.

Western Blot and Immunofluorescence Detection—Whole cell lysates were obtained by addition of Tris-2%SDS pH: 6.8 buffered solution to the cell cultures at the end of the treatments. Protein extracts were denatured by heat shock at 95 °C for 3 min and quantified by the Lowry Assay (Bio-Rad). Equal amounts of protein were electrophoresed by SDS-PAGE, transferred to polyvinylidene difluoride membranes (Amersham Biosciences), and probed with specific antibodies (supplemental Table S1). For immunofluorescence detection, cells were cultured in 4-well plates (NunClon) and fixed by either incubation with paraformaldehyde 4% for 10 min or by 100% methanol for 5 min. Cells were rinsed, permeabilized, and stained as described previously (31). For apoptosis quantification, cells showing cleaved caspase-3 expression and nuclear fragmentation were counted as apoptotic. At least, 100 cells were analyzed per condition and values are mean ± S.E. of three independent experiments.

DNA Integrity Assay—Cells were pelleted at the end of each treatment and frozen at −80 °C. Pellets from the same experiment were processed at once. They were diluted in 40 μl of sterile phosphate-buffered saline, mixed with 40 μl of melted 1% low melting agarose (Sigma) in 0.5× TBE (45 mM Tris pH 8.3, 45 mM boric acid, 1.0 mM EDTA). Each mixture was poured into a block caster and let to solidify. Each agarose block was submerged into 1 ml of lysis buffer (1% lauryl sarcosil, 0.5 M EDTA, 10 mM Tris, pH 8, 100 μg/ml proteinase K) at 50 °C during 24 h in mild agitation, and rinsed twice with 0.5× TBE for 1 h at room temperature. For analysis of DNA high molecular weight degradation, the blocks were then laid into wells of a 1% agarose, 0.5× TBE gel (CHEF grade, Sigma). Pulse field electrophoresis was performed in a CHEF DR-II system (Bio-Rad) set to the following protocol: run time, 14 h; switch time from 5 to 50 s; voltage gradient, 6 V/cm. Initial lysis buffer was further processed to analyze DNA low molecular weight degradation. Briefly, 1 ml of lysis buffer from DNA extraction was mixed with 1 ml of ethanol, kept at −20 °C for 18 h, and centrifuged. The pellet was rinsed with 70% ethanol, centrifuged again, and the final DNA pellet was diluted in 20 μl of 10 mM Tris, pH 8, 1 mM EDTA, and 10 μg/ml RNase. Conventional 2% agarose-gel electrophoresis was performed. Gels were stained with SYBR Safe (Molecular Probes), visualized by UV exposure and recorded with a Kodak DC290 digital camera.

Overexpression and Detection of FLAG-tagged EndoG in Cardiomyocytes—To test the hypothesis that EndoG translocates from mitochondria to cytosol during ischemia in cardiomyocytes, the coding sequence of EndoG contained in the mouse IMAGE clone 5029633 (MRC Gene Service) was PCR-amplified flanked by the EcoRI and XbaI sites and was subcloned into the pCDNA3.1 expression plasmid (Invitrogen) in-frame and upstream to a FLAG tag. Cardiomyocytes were transfected by the Lipofectamine system (Invitrogen) and overexpressed EndoG-FLAG was detected by immunofluorescence and Western blot with an anti-FLAG antibody (Sigma).

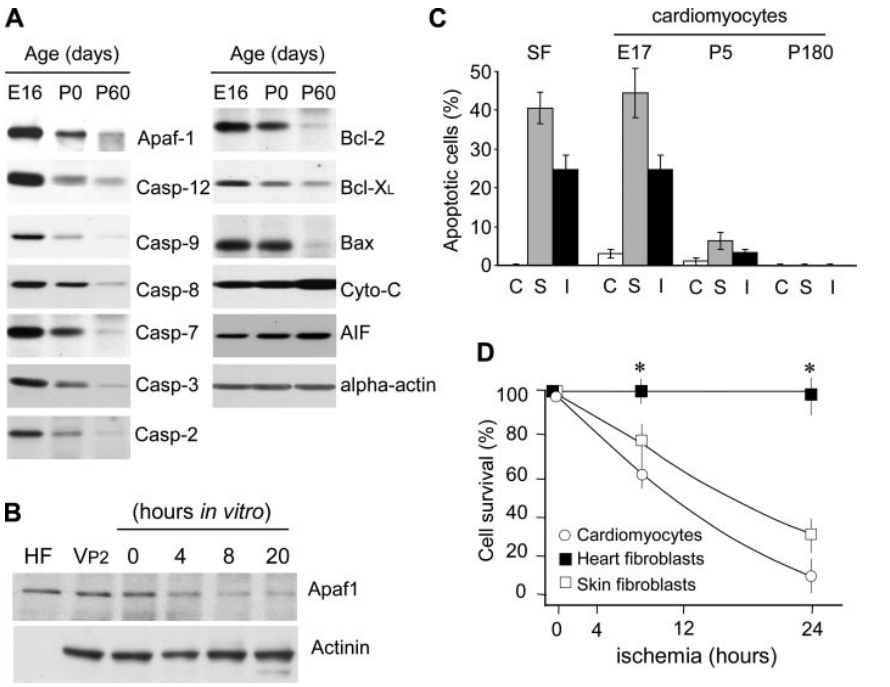

FIGURE 1. **Silencing of caspase expression during cardiomyocyte differentiation *in vivo* and *in vitro* correlates with a reduction in ischemia-induced caspase activation.** *A*, expression of caspases, cytochrome *c*, Bcl-2-related proteins, and Apaf-1 was detected in SDS extracts of ventricles from embryonic day 16 (E16), neonatal (P0), and 60-day-old rats (P180). *B*, Apaf-1 protein expression in protein extracts of cardiac fibroblasts (*HF*), ventricle of a 2-day-old rat (*VP2*), and isolated cardiomyocytes from 3-day-old pups, at 0–20 h *in vitro*. *C*, cells expressing cleaved caspase-3 and presenting fragmented nucleus were counted as apoptotic in cultures of skin fibroblasts, and embryonic, neonatal and adult cardiomyocytes. *C*, control; *S*, staurosporine 1 μM 16 h; *I*, ischemia 16 h). Skin fibroblasts (*SF*) were used as positive control. *D*, cell viability during ischemia in postnatal cardiomyocytes, cardiac, and dermal fibroblasts. Values are percentage of living cells *versus* cells in plates counted at time 0. *, $p < 0.01$ *versus* cardiomyocytes and skin fibroblasts. Data are means ± S.E. of three independent experiments made in triplicates.

Gene Knockdown by Small Hairpin RNA Interference (shRNAi) using Lentiviral Vectors—Specific 19 nucleotide sequences were chosen for each gene (AIF, EndoG) using the free RNAi design interfaces available at the Promega and Invitrogen web sites. Primer couples were designed for cloning in the pSUPER plasmid (Oligoengine) using the BglII and HindIII sites. Primers were designed against the sequences 5′-GGCAACATGGTGAAACTTA-3′ for rat AIF and 5′-GGAACAACCTTGAGAAGTA-3′ for rat EndoG. Then, an EcoRI/ClaI fragment containing the H1 promoter for the RNA polymerase III and the shRNAi sequence was cut from pSUPER and subcloned into the pLVTHM plasmid (from Dr. Trono, Geneva). For each gene, we designed a couple of primers carrying two nucleotide changes from the original sequence that were initially used to verify the specificity of the shRNAi constructs. Each specific shRNAi vector was transfected into HEK293T cells together with the plasmids psPAX2 and pMD2G (from Dr. Trono), using the polyethyleneimine transfection method (Sigma). Transfection efficiency was analyzed by detection of the enhanced green fluorescence protein (EGFP) expression, whose coding sequence is contained in the pLVTHM plasmid and driven by an IRES sequence. The 293T medium was collected after 48 h of transfection and centrifuged at 50,000 × *g* for 3 h. The final viral pellet was diluted in sterile phosphate-buffered saline plus 2% bovine serum albumin and titrated by transduction of cardiac fibroblasts before treating cardiomyocytes. Cardiomyocytes were transduced 4 h after seeding. Efficiency of transduction was estimated to be over 80% by detection of EGFP expression at 48 h. The capability of the constructs for repressing each gene was routinely tested by Western blot for AIF and by RT-PCR from total RNA for EndoG, as described below. The maximum efficacy of the shRNAi plasmids for reducing endogenous expression of the genes was determined to reach a plateau from 4–6 days after transduction, depending on the gene. Therefore, treatment of cardiomyocytes was started always 6 days after transduction, *i.e.* after seeding. All experiments carried out with transduced cardiomyocytes were repeated at least four times.

Detection of EndoG Transcript by RNA Reverse Transcription Coupled to Standard PCR—Gene expression was analyzed by reverse transcription coupled to PCR (RT-PCR) from total RNA extracts as reported for other genes (31). Conventional PCR was performed in a GeneAmp PCR System 2700 thermocycler (Applied Biosystems), using an annealing temperature of 55 °C, with the primers forward: 5′-GACTTCCACGAGGACGATTC-3′, and reverse: 5′-AAGCTGCGGCTGTACTTCTC-3′, producing an amplicon of 218 bp. Control for cDNA input in the amplification reaction performed with a couple of primers amplifying a fragment of the "upstream of n-Ras" (unr) gene. PCR products were migrated in 2% agarose gels and visualized by SYBR Safe staining.

Statistical Analysis—Data are expressed as mean ± S.E. of three independent experiments. The significance of differences among means was evaluated using the Student's *t* test. A value of $p < 0.05$ was considered significant.

RESULTS

Down-regulation of the Caspase-dependent Death Pathway during Cardiac Development Is Associated with a Decrease in Ischemia-induced Caspase Activation in Cardiomyocytes—Expression of the main regulators of caspase-dependent cell death decreased with age in the rat myocardium (Fig. 1*A*). Apoptotic gene repression took place also *in vitro* in postnatal cardiomyocytes (Fig. 1*B*). Experimental ischemia (ischemia, hereafter) was achieved by culturing cells inside a hypoxic chamber (31) in a Tyrode's solution without serum and glucose that becomes acidic during hypoxia (final pH 6.1–6.4). We used skin fibroblasts as positive control for the activation of caspases in this setting (31). Executioner caspase-3 activation was observed in embryonic cardiomyocytes and skin fibroblasts, but not in postnatal and adult cardiomyocytes during ischemia or stauro-

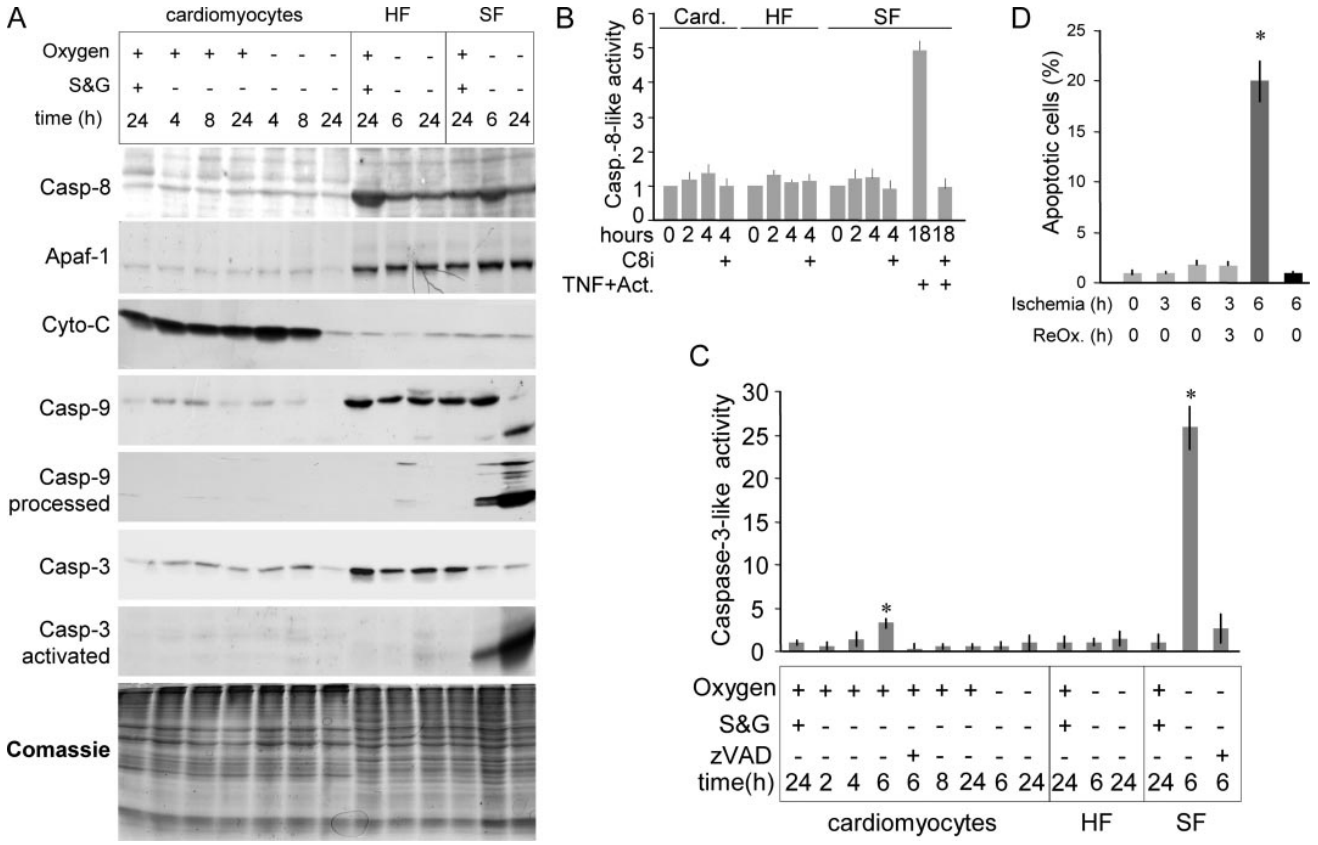

FIGURE 2. **Lack of caspase re-expression in ischemic cardiomyocytes and relevance of the mitochondrial pathway in ischemia-induced fibroblast apoptosis.** *A*, detection of caspases, their cleaved (active) products, and some regulators of apoptosis, in total protein extracts of postnatal day 5 cardiomyocytes, cardiac (*HF*), and skin fibroblasts (*SF*), cultured in presence or absence of serum and glucose (*S&G*) and oxygen. *B*, initiator caspase-8 activity in extracts of ischemic cardiomyocytes (*Card.*), cardiac (*HF*), and dermal fibroblasts (*SF*), in the presence or absence of a caspase-8 inhibitor (*C8i*). Skin fibroblasts treated with TNF-α+actinomycin were used as positive control. *C*, executioner caspase activity measured in extracts of serum and glucose-deprived (*S&G*), or serum-, glucose-, and oxygen-deprived (ischemic) postnatal cardiomyocytes, heart fibroblasts (*HF*), and skin fibroblasts (*SF*). zVAD, pan-caspase inhibitor z-VAD.fmk 100 μM; *, $p < 0.01$ *versus* control. Caspase activities are expressed as fold increase *versus* control value. *D*, adult cardiomyocytes (*gray bars*), embryonic cardiomyocytes (*dark gray bar*), and heart fibroblasts (*black bar*) expressing processed caspase-3 were counted as apoptotic, at several time points during ischemia and reoxygenation. Data are apoptotic cells *versus* total cells in each well and means were calculated from 4 wells per treatment. *, $p < 0.01$ *versus* adult cardiomyocytes.

sporine treatment (Fig. 1*C* and supplemental Figs. S1–S3). However, ischemia induced cell death to a similar extent in postnatal cardiomyocytes and skin fibroblasts, whereas cardiac fibroblasts survived to the ischemic period (Fig. 1*D*). On this account, we have previously reported that cardiac fibroblasts are resistant to apoptosis because of their strong expression of the antiapoptotic factor Bcl-2 (31). These data establish a correlation between the silencing of the apoptotic genes during myocardial development and the reduction in the competence of postnatal cardiomyocytes to activate apoptosis.

Lack of Caspase Re-expression and Caspase Activity in Cardiomyocytes during Ischemia and Relevance of the Mitochondrial Pathway in Ischemia-induced Fibroblast Apoptosis—Expression of the initiator caspases-8 and -9, the postmitochondrial regulator Apaf-1, and the executioner caspase-3, were significantly less expressed in cultured postnatal cardiomyocytes than in cardiac and dermal fibroblasts (Fig. 2*A*). Importantly, myocardial expression of these genes was not up-regulated during ischemia (Fig. 2*A*). Processing of caspase-9, an initiator caspase in the mitochondrial pathway, occurred in skin fibroblasts and, later in cardiac fibroblasts, but not in cardiomyocytes (Fig. 2*A*). Initiator caspase-8-like activity was measured as an indication of the role of the death receptor-dependent pathway during ischemic cell death. Caspase-8 activity was not detected in ischemic cardiomyocytes, cardiac, and dermal fibroblasts before caspase-3 was engaged (Fig. 2*B*). Executioner caspase activity was not detected in ischemic cardiomyocytes but was evident in skin fibroblasts (Fig. 2*C*). These results suggest that a low expression of the caspase-dependent machinery in postnatal cardiomyocytes is maintained during ischemia, thus limiting the impact of caspase activation. Furthermore, executioner caspase processing was not substantially enhanced when ischemic adult cardiomyocyte cultures (pH 6.1–6.4) were reoxygenated and cultured in standard medium (pH 7–7.5) (Fig. 2*D*). However, ischemia activated the mitochondrial pathway of apoptosis in proliferating cell types such as fibroblasts (Fig. 2*A*).

The Pattern of DNA Degradation Induced during Ischemia in Cardiomyocytes Suggests the Participation of Caspase-independent Mechanisms—Caspase-dependent cell death induces a characteristic pattern of DNA degradation related to the activity of endonucleases that cleave DNA between nucleosomes, rendering fragments of low molecular weight, the so-called

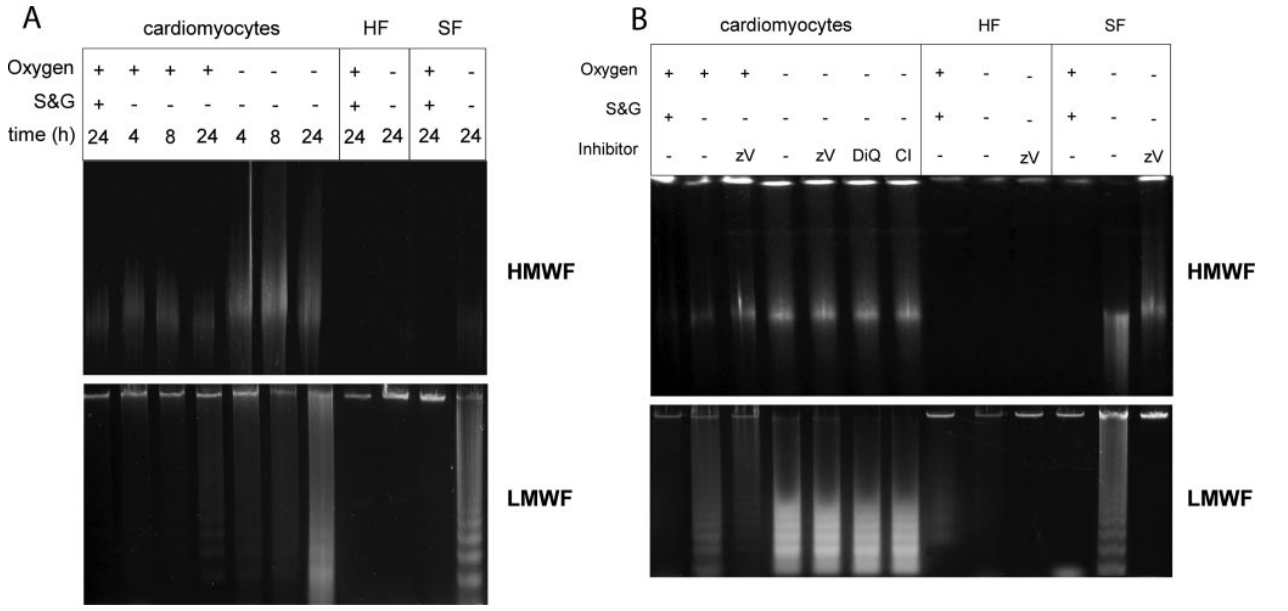

FIGURE 3. **Ischemia induces caspase-independent DNA fragmentation in postnatal cardiomyocytes.** Simultaneous analysis of high and low molecular weight fragmentation (*HMWF* and *LMWF*) in DNA obtained from cardiomyocytes, heart-derived (*HF*), and skin (*SF*) fibroblasts. *A*, time course of DNA degradation in cultures exposed to the presence or absence of oxygen and/or serum and glucose (*S&G*). *B*, DNA fragmentation at 18 h of serum and glucose (*S&G*) deprivation or ischemia in cardiomyocytes, cardiac (*HF*) and skin (*SF*) fibroblasts, with or without 100 μM pan-caspase inhibitor z-VAD.fmk (*zV*), 30 μM PARP-1 inhibitor DIQ, or 100 μM calpain inhibitor MDL 28170 (*CI*). Results in this figure are representative of three independent experiments.

DNA laddering. Caspase-independent PCD can induce high molecular weight fragmentation of DNA, mainly in fragments of ~50 kb (14), and/or low molecular weight fragmentation, which appears as a smear after electrophoresis, because of the ability of the caspase-independent DNAses, such as EndoG, to cleave inside the nucleosomes (33). In aerobic conditions, serum and glucose deprivation for 20 h induced two types of DNA degradation in cardiomyocytes. A faint DNA low molecular weight laddering (Fig. 3*A*, *lower panel*) that was partially reverted by the pan-caspase inhibitor z-VAD.fmk (Fig. 3*B*, *lower panel*), and high molecular weight fragmentation (Fig. 3*A*, *upper panel*), which was not blocked by z-VAD.fmk (Fig. 3*B*, *upper panel*). During ischemia, *i.e.* serum and glucose deprivation in hypoxia and acidosis, cardiomyocyte DNA was fragmented rendering a low molecular weight smear in agarose gels. Upon ischemia, DNA smear was not inhibited by the caspase inhibitor z-VAD.fmk. PARP-1 has been involved in ischemia-induced neuronal cell death (34); however, ischemia-induced cardiac DNA damage was not blocked by the two PARP inhibitors DIQ and DPQ. In addition, although calpains have been shown to trigger the release of AIF *in vitro* (35), addition of the pan-calpain inhibitor MDL 28170 did not prevent DNA high molecular weight degradation in ischemic cardiomyocytes (Fig. 3, *A* and *B*). Furthermore, high molecular weight fragmentation of DNA, compatible with the activity of caspase-independent pathways, was rapidly activated within a few hours of ischemia (Fig. 3*A*, *upper panel*), and was not inhibited neither by z-VAD.fmk, nor by DIQ. In contrast, DNA degradation was not observed in ischemic heart fibroblasts after 24 h of treatment, in agreement with our previous results (31). Ischemic skin fibroblasts showed a canonical pattern of DNA low molecular weight degradation, which was completely abolished by z-VAD.fmk (Fig. 3, *A* and *B*). Therefore, the pattern of DNA degradation during cardiomyocyte ischemia and its execution in the presence of caspase inhibitors suggest the predominant role of a caspase-independent mechanism of cell death.

Ischemia Induces Cytochrome c, Truncated AIF, and EndoG Release from Cardiac Mitochondria in the Absence of Caspase Activation—We have previously reported that staurosporine does not induce caspase activation in cardiomyocytes despite cytochrome *c* translocation, and that overexpression of Apaf-1 partially antagonizes this resistance (4). Therefore, we analyzed the release of proapoptotic mitochondrial factors during ischemia in cardiomyocytes. Cytochrome *c* translocation was detected during ischemia in embryonic and postnatal cardiomyocytes (Fig. 4*A*, *panels B*, *D*, *F*, and *H*, and Fig. 4*C*); however, this event was followed by caspase-3 cleavage (activation), and by nuclear fragmentation in embryonic (Fig. 1*C* and Fig. 4*A*, *panels C* and *D*) but not in postnatal cardiomyocytes (Fig. 4*A*, *panels G* and *H*).

AIF and EndoG are mitochondrial proteins that have been reported to act as executioners of PCD after their translocation to the cytosol (14, 27). AIF was released from mitochondria during cardiac ischemia (Fig. 4, *B* and *C*), but cytosolic AIF was smaller in size than mitochondrial AIF, as detected with an antibody raised against the C terminus of the protein (Fig. 4*C*). This is in agreement with the recently proposed apoptosis-induced cleavage of AIF at the N terminus, which allows its detachment from the inner mitochondrial membrane and subsequent translocation (36). This cleavage has been proposed to depend *in vitro* on calpain and/or cathepsin activity (35, 36). However, pretreatment of cardiomyocytes with the pan-cal-

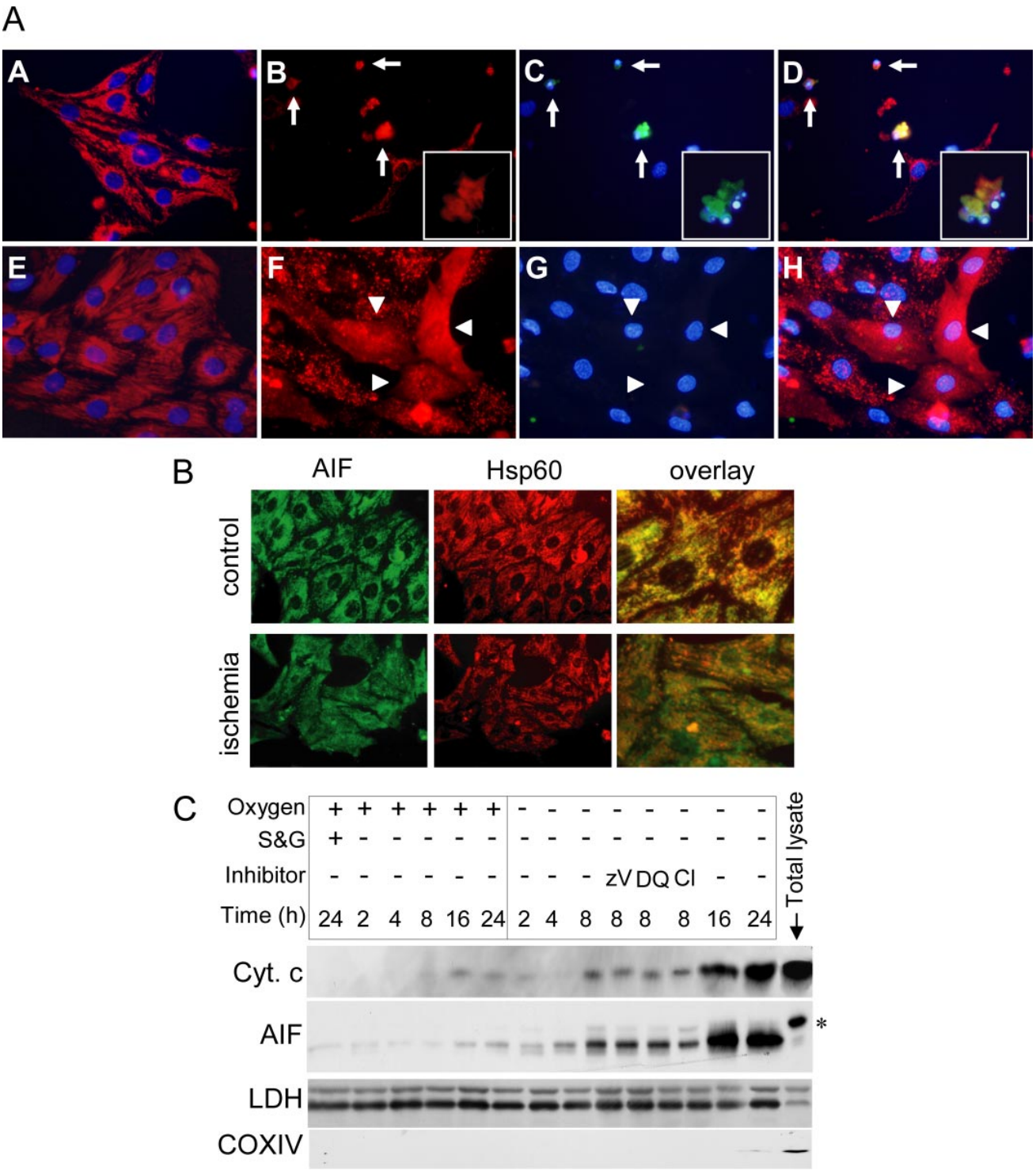

FIGURE 4. **Ischemia triggers the translocation of mitochondrial regulators of programmed cell death cytochrome *c* and truncated AIF in cardiomyocytes independently of caspase activation.** *A*, immunofluorescence staining of cytochrome *c* (*red*) and cleaved caspase-3 (*green*), in embryonic (*panels A–D*) and postnatal (*panels E–H*) cardiomyocytes cultured in standard conditions (*panels A* and *E*), or after 6 h of ischemia. Chromatin is shown in *blue*. *Panels B* and *F* correspond to cytochrome *c*; *panels C* and *G* are cleaved caspase 3 expression and nuclear chromatin; *panels D* and *H* are overlay compositions. *Arrows* point to cells undergoing cytochrome *c* release, caspase-3 activation and nuclear fragmentation, and *arrowheads* point to cells undergoing cytochrome *c* release without caspase activation. *Inset*, high magnification of a representative apoptotic embryonic cell. *B*, AIF (*green*) and the mitochondrial marker Hsp60 (*red*) in control cardiomyocytes (*control*), or after 18 h of serum, glucose, and oxygen deprivation (*ischemia*). Overlay of AIF and Hsp60 images, *orange* means co-localization and *green* indicates AIF translocation. *C*, cytochrome *c* (*Cyt.c*) and AIF in cytosolic extracts of serum and glucose (*S&G*)-deprived, and ischemic cardiomyocytes. *zV*, 100 μM pan-caspase inhibitor z-VAD.fmk; *DQ*, 30 μM PARP inhibitor; *Cl*, 100 μM calpain inhibitor MDL 28170. Cytosolic Lactate dehydrogenase (*LDH*) was used as a loading control, and cytochrome *c* oxidase subunit IV (*COXIV*) was detected as a marker for mitochondrial contamination. *, full-length AIF. Each image in this figure is representative of three independent experiments.

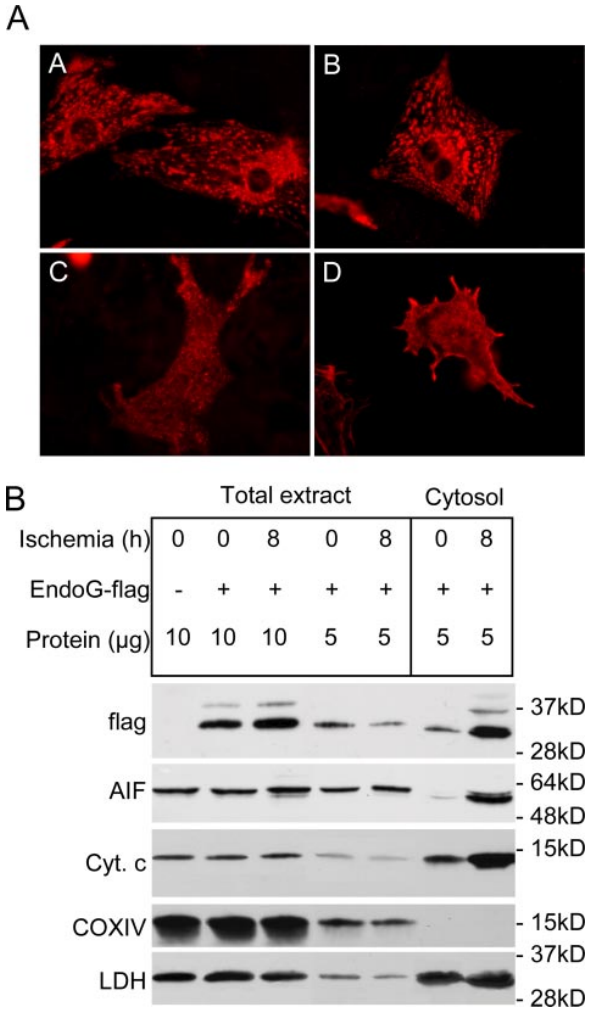

FIGURE 5. **Ischemia induces EndoG release from cardiac mitochondria.** EndoG-FLAG was transfected in cardiomyocyte cultures as described under "Experimental Procedures." *A*, expression of exogenous EndoG-FLAG was examined by immunofluorescence with an anti-FLAG antibody in control cardiomyocyte cultures 3 days after transfection (*panels A* and *B*), and in cardiomyocytes exposed to 16 h of ischemia the 3rd day (*panels C* and *D*). *B*, EndoG-FLAG was detected in SDS-PAGE-electrophoresed total protein extracts and cytosolic protein extracts in control and ischemic cardiomyocytes. AIF and cytochrome *c* were detected to check the effectiveness of the ischemic treatment in releasing endogenous mitochondrial proteins. COXIV is a mitochondrial marker and LDH is a marker for the enrichment in cytosolic proteins. The images are representative of three independent experiments.

pain inhibitor MDL 28170 did not prevent AIF cleavage and its release during ischemia (Fig. 4*C*).

Ischemia-induced EndoG translocation was investigated by immunofluorescence and by Western blot with three commercial antibodies (supplemental Table S1). However, our results suggested that these antibodies are not specific when used on rat samples. We therefore constructed a plasmid for the overexpression of mouse EndoG with a C-terminal FLAG tag. EndoG-FLAG tracking demonstrated that EndoG had a punctuated pattern that excluded the nucleus under basal conditions, which is consistent with a mitochondrial localization (Fig. 5*A*, *panels A* and *B*). However, EndoG pattern changed during ischemia in accordance with its translocation to the cytosol and nucleus (Fig. 5*A*, *panels C* and *D*). We verified by Western blot that overexpressed EndoG had the expected size of 32 kDa, and no cleavage bands were observed in ischemic cardiomyocyte extracts (Fig. 5*B*), suggesting that EndoG translocation was not dependent on its cleavage, contrary to AIF (Fig. 5*B*). Taken together, these results demonstrate that mitochondrial proteins cytochrome *c*, truncated AIF and EndoG are translocated to the cytosol in ischemic postnatal cardiomyocytes, yet caspases and calpains are not essential for their release.

EndoG Is an Important Executor of DNA Processing during Ischemia in Cardiomyocytes—The pattern of DNA cleavage in ischemic postnatal cardiomyocyte indicated the participation of caspase-independent mechanisms. AIF and EndoG have been suggested to execute cell death in the absence of caspase activation (14, 27). This prompted us to test if AIF and EndoG, which were released from mitochondria during ischemia, were responsible for cardiac cell death in this model. Cardiomyocytes were transduced with lentiviral vectors carrying constructs for small hairpin-based RNA interference (shRNAi) of either protein. Six days after transduction, cardiomyocytes were exposed to ischemia (see "Experimental Procedures"). The effectiveness of AIF shRNAi vector was evidenced by the decrease in AIF protein levels measured in cardiomyocyte total lysates (Fig. 6*A*). A time course analysis of AIF expression in cardiomyocytes transduced with the AIF-specific shRNAi plasmid showed that maximal reduction of AIF expression was achieved at day six after infection (Fig. 6*A*, *upper panel*). Therefore, all experiments were carried out at day six of transduction. AIF expression was reduced up to 80% in cardiomyocytes (Fig. 6*A*, *lower panel*). Reduction in EndoG expression was checked by RT-PCR from total RNA extracts to confirm the efficacy of the EndoG-specific shRNAi construct (Fig. 6*B*). Reduction in EndoG expression, but not in AIF levels, correlated with an important reduction of DNA damage (Fig. 6*C*). A faint ladder pattern was observed after electrophoresis of DNA from ischemic cardiomyocytes in which EndoG expression was reduced. This faint ladder was inhibited by z-VAD.fmk addition, suggesting a very low contribution of caspases (Fig. 6*C*). Interestingly, ischemia-induced DNA high molecular weight fragmentation was not blocked in cells with reduced EndoG expression, suggesting the presence of another yet unidentified AIF-independent, EndoG-independent nuclease working in the absence of caspase-activation. In contrast, AIF was essential for the ischemia-induced high molecular weight processing of DNA in skin fibroblasts (Fig. 6*D*), whereas the caspase-dependent DNase activity, but not EndoG, was involved in the low molecular weight DNA cleavage in these cells (Fig. 6*D*).

To ascertain whether the reduction in EndoG expression favored cardiomyocyte cell survival during ischemia, analysis of cell survival in scrambled-transduced, AIF-deficient and EndoG-deficient cardiomyocytes was performed. Neither AIF nor EndoG knockdown prevented ischemia-induced cardiac cell death, although reduction in AIF expression substantially blocked cardiac cell recovery during reoxygenation (Fig. 7). Altogether, these results reveal a main role of caspase-independent mechanisms in cardiomyocyte ischemic cell death, and identify EndoG as an essential executor of ischemia-induced DNA processing in these cells.

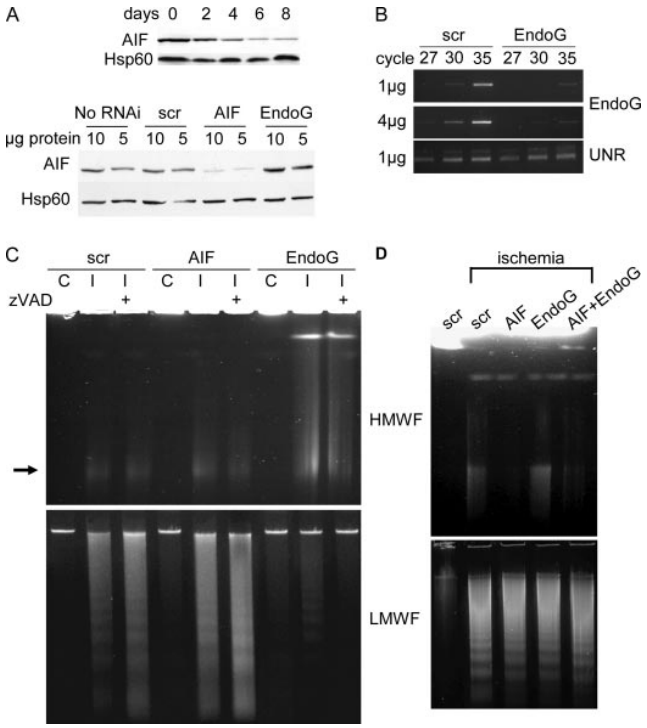

FIGURE 6. **Reduction of EndoG expression protects differentiated cardiomyocytes from ischemia-induced DNA fragmentation.** *A*, time course of AIF expression in cardiomyocytes transduced with an AIF-specific shRNAi construct (*upper panel*), and expression of AIF in 10 and 5 μg of protein from total extracts of normal cardiomyocytes (*No RNAi*), scrambled-transduced (*scr*), AIF-deficient (*AIF*) and EndoG-deficient (*EndoG*) cardiomyocytes, 6 days after infection. Hsp60 was used as loading control. *B*, EndoG transcript levels in scrambled-transduced cardiomyocytes (*scr*) or EndoG-deficient cardiomyocytes (*EndoG*). RT-PCR was performed from 1 and 4 μg of total RNA and equal volumes of the PCR product extracted at PCR cycles 27, 30, and 35 were loaded and electrophoresed in a SYBR Safe-stained 3% agarose gel. PCR reaction from 1 μg of total RNA was conducted in parallel for amplification of the *unr* transcript, used as loading control. *C*, DNA high and low molecular weight fragmentation (*HMWF* and *LMWF*) induced by exposure to 18 h of ischemia with (+) or without 100 μM z-VAD.fmk (*zV*), in scrambled-transduced (*scr*), AIF-deficient (*AIF*), and EndoG-deficient (*EndoG*) cardiomyocytes. *Arrow* indicates band at 50 kb. *D*, DNA high and low molecular weight fragmentation (*HMWF* and *LMWF*) induced by exposure to 18 h of ischemia in scrambled-transduced (*scr*), AIF-deficient (*AIF*), EndoG-deficient (*EndoG*), and AIF/EndoG double-knockdown skin fibroblasts. The images are representative of three independent experiments.

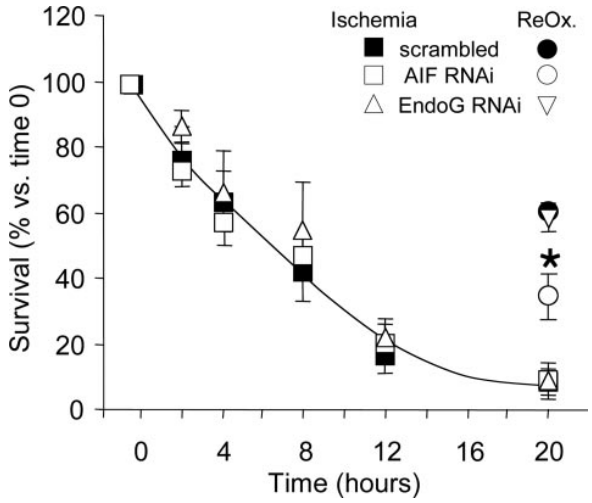

FIGURE 7. **EndoG activity does not affect cardiomyocyte viability during ischemia and AIF limits cell death during reoxygenation.** Cardiomyocyte viability in ischemic cultures at several time points between 0 and 20 h (*Ischemia*). Survival was also counted in cardiomyocytes cultured for 2 h in ischemic conditions and then reoxygenated in standard medium for 18 h (*ReOx.*). Data are mean ± S.E. of three independent experiments made in duplicates. *, $p <$ 0.05 AIF-deficient *versus* scrambled and EndoG-deficient at the same time point.

DISCUSSION

The results presented here show that the silencing of the caspase-dependent machinery during cardiac differentiation (both *in vivo* and *in vitro*) accounts for the lack of caspase-dependent cell death during experimental ischemia. Our data also prove that the main pathway for DNA processing in cardiac cells involves the caspase-independent release of the mitochondrial endonuclease EndoG, which helps other caspase-independent, AIF-independent, DNAses in the cleavage of cardiac DNA. Furthermore, our results suggest the presence of a previously not described caspase-independent nuclease activity that induces high molecular weight fragmentation of DNA in ischemic cardiomyocytes. Finally, the prosurvival role of AIF in cardiomyocytes during reoxygenation is described.

During an ischemic insult, postnatal cardiomyocytes do not activate caspases as readily as other cell types (9, 37). However, the underlying molecular mechanisms are not well understood because cardiac cells have been assumed to express a functional caspase-dependent pathway. Several molecules have been described to protect cardiomyocyte mitochondria by blocking cytochrome *c* release, and have been proposed to be apoptotic blockers in cardiomyocytes. Heat shock proteins have been suggested to be endogenous protectors against ischemic damage in cardiomyocytes (38, 39) by protecting mitochondrial function (40). Experimental overexpression of Bcl-2 or Bcl-X_L, which block cytochrome *c* translocation, have been reported to be cardioprotective during ischemia/reoxygenation (41–43). Accordingly, the hearts of mice deficient for Bax, which induces cytochrome *c* release, are partially protected during ischemia (44). Apoptosis Repressor with Caspase Recruitment Domain (ARC) is highly expressed in the heart (45), and protects this organ against ischemic-induced cell death (46). ARC blocks cytochrome *c* release by interfering with Bax (47, 48). From the above-mentioned observations, it has been assumed that activation of the mitochondrial apoptotic pathway leading to executioner caspase activation is relevant in the cardiac ischemic damage. However, caspase activity was not measured (46), or it was only analyzed in the heart-derived cell line H9c2 (45, 47, 48). Importantly, heart-derived cell lines H9c2 and HL-1 (49) are not terminally differentiated cells and, contrary to cardiomyocytes, express all the apoptotic regulators.[7]

Furthermore, it is unknown why in conditions where caspase-dependent programmed cell death should be engaged (*i.e.* after cytochrome *c* release (50)), there was only low caspase activation in cardiomyocytes (51). What blocks cardiomyocyte apoptosis after cytochrome *c* release? We have previously described that cardiomyocytes lack significant Apaf-1 expression (4), and this could hamper the activation of caspase-9 after

[7] N. Bahi, J. Zhang, and D. Sanchis, unpublished data.

cytochrome *c* release. A recent report proposes that the low expression of Apaf-1 helps inhibitor of apoptosis proteins (IAPs) to better counteract executioner caspase activation in cardiomyocytes, which were suggested to express caspases at a similar level than fibroblasts (52). Contrary to this work, the data reported here demonstrate a global down-regulation of the whole caspase-dependent machinery in the heart during development. Reduction in caspase expression is evident when comparing pure isolated embryonic and adult cardiomyocytes,[7] suggesting that most of the caspase expression in neonatal cardiomyocyte cultures comes from dividing cardiac fibroblasts, which usually contaminate cardiomyocyte cultures. In addition, our results discard an inhibition of caspase activation by ischemia-induced acidosis, because fibroblasts and embryonic cardiomyocytes do activate caspases in this setting, even at pH 6.1. Therefore, we propose that differentiation-related repression of the whole caspase-dependent machinery accounts for postnatal cardiac protection against caspase activation. We hypothesize that down-regulation of the apoptotic machinery during differentiation can be a mechanism for reducing caspase-induced cell damage by accidental cytochrome *c* translocation due to the high number of mitochondria and the constant change of cardiomyocyte volume during the contraction/relaxation cycle.

Our results demonstrate that cytochrome *c* translocation during cardiac ischemia is not linked to the caspase-dependent death pathway, contrary to the dogma that has been established in recent years. Nevertheless, mitochondrial damage is involved in ischemia-induced cardiomyocyte cell death and hampers cardiac recovery during reoxygenation. In this respect, truncated-AIF translocation from mitochondria was detected in ischemic cardiomyocytes. Of note, pharmacological calpain inhibition did not prevent ischemia-induced AIF cleavage and release. This fact discards an essential role of calpains in AIF translocation and release in ischemic cardiomyocytes. In our experimental model, AIF release did not account for ischemia-induced cardiac DNA damage. Furthermore, we show that AIF depletion hampers cardiac survival during reoxygenation. Importantly, the ischemic lesion is bigger in the AIF-defective Harlequin mouse (25). In addition, we have recently shown that AIF is not related to DNA damage in staurosporine-treated neuroblastoma cells (53). Therefore, our data suggest that AIF role in DNA cleavage depends on the cell type and does not support a relevant role for AIF in cardiac DNA processing.

The present study also reveals that EndoG is the main contributor for the DNA low molecular weight degradation in ischemic cardiomyocytes. Interestingly, two recent works reported no obvious effects on cell death and DNA degradation in EndoG-deficient mice (29, 30). However, DNA integrity analysis was studied in splenocytes, which otherwise activate caspase-dependent apoptosis (29). It must be taken into account that Li *et al.* showed the EndoG role in mammalian apoptotic DNA degradation in fibroblasts deficient for caspase-activated DNase (27). Furthermore, here we show that EndoG silencing in primary skin fibroblasts, which express caspases, does not induce a reduction in DNA cleavage. Taken together, these results suggest that EndoG cleaves DNA in systems where caspases are absent, irrespective of the death stimulus, and could be the final step of DNA processing in postmitotic cells.

Finally, our results show that high molecular weight fragmentation of DNA is caspase-independent in dividing fibroblasts and postmitotic cardiomyocytes and that AIF is involved in this step of DNA degradation only in proliferating skin fibroblasts but not in cardiomyocytes. Indeed, a functional genomic approach in *C. elegans* has highlighted the involvement of many genes in the final apoptotic DNA degradation process, which could be controlling the final cell fate (54). Therefore, we are currently investigating the identity of the nuclease activities involved in the ischemia-induced DNA high molecular weight fragmentation.

In summary, the involvement of caspases in cardiac cell death has been based on the assumption that the caspase-dependent machinery is expressed in differentiated cardiomyocytes and has been inferred mainly from the experimental evidence of cytochrome *c* translocation and the cleavage of DNA rendering 3′-OH ends, which are detected by the TUNEL assay, a characteristic feature of the caspase-dependent DNase CAD/DFF40, but that can be also produced by EndoG (55). In contrast, here we show that cardiomyocytes reduce the expression of the caspase-dependent cell death machinery during differentiation and that these proteins are not up-regulated during ischemia. Our results show that, after differentiation, caspase-independent mechanisms substitute for caspases in the ischemic cell damage of cardiomyocytes. Furthermore, our data disclose the essential role of EndoG, which works in concert with a yet unidentified nuclease activity, as an important executor of caspase-independent DNA cleavage in differentiated cardiomyocytes.

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Supplementary Table 1. Antibodies

Antigen	Provider	Catalogue Number	Application
α-actinin sarcomeric	SIGMA	A7811	WB
AIF	SIGMA	A7549	IF / WB
Apaf-1	ALEXIS	ALX-804-349	WB
Bax	Cell Signaling	2772	WB
Bcl-2	NeoMarkers	MS-598	WB
Bcl-XL	BD	610211	WB
Caspase-3	Cell Signaling	9665	WB
Caspase-3 cleaved	Cell Signaling	9664S	IF / WB
Caspase-7	MBL	JM-3024-100	WB
Caspase-8	Stressgen	AAP-128	WB
Caspase-9	Cell Signaling	9508	WB
Caspase-9 cleaved	Cell Signaling	9506	WB
Caspase-12	SIGMA	C7611	WB
COXIV	Molecular Probes	A-21348	WB
Cytochrome c	BD	556432	IF
Cytochrome c	BD	556433	WB
Endonuclease G	ProSci	PSC-3035	WB/IF
Endonuclease G	Santa Cruz	sc-26924	WB
Endonuclease G	SIGMA	E5654	WB
Flag	SIGMA	F3165	IF/WB
Hsp60	BD Transduction	H99020	IF / WB
LDH	Rockland	100-1173	WB

Applications used were IF: Immunofluorescence, WB: Western blot.

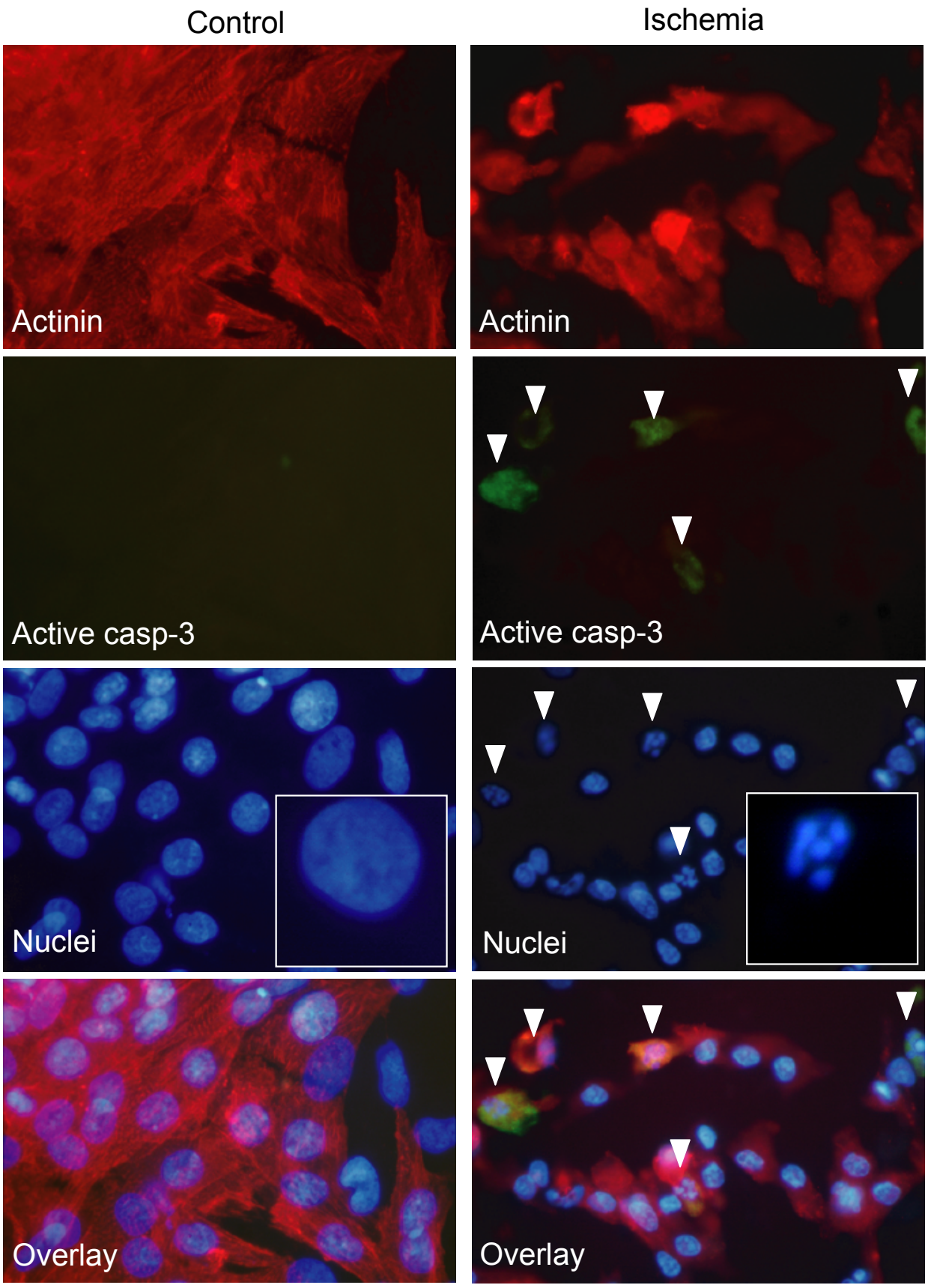
SUPPLEMENTARY FIGURE LEGENDS

Fig. 1. *Immunofluorescence detection of caspase-3 activation and nuclear fragmentation in embryonic cardiomyocytes.* Embryonic cardiomyocytes were isolated from rat day 17 embryos, plated on collagen-coated plates, changed 20 hours later to the ischemic medium and cultured for 16 hours under hypoxic conditions (ischemia), or left untreated (control). Cells were fixed with PFA 4% and immunostained for cardiac-specific actinin (red), cleaved caspase-3 (green). Chromatin was stained with the Hoechst dye (blue). Arrow heads point to cardiomyocytes undergoing caspase-dependent cell death. Insets: higher magnification of nuclear morphology. The images are representative of three independent experiments.

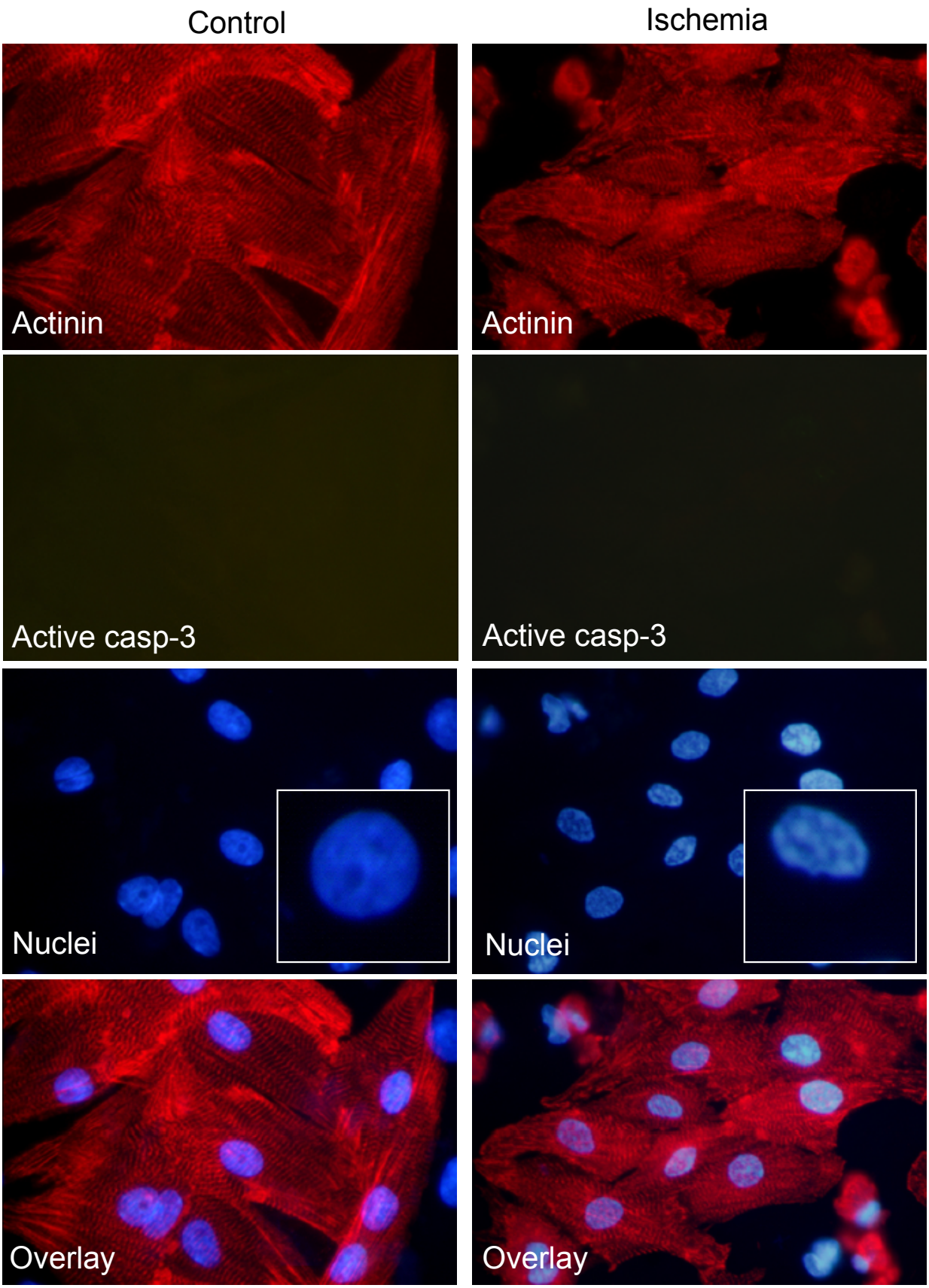
Fig. 2. *Immunofluorescence detection of caspase-3 activation and nuclear fragmentation in neonatal cardiomyocytes.* Neonatal cardiomyocytes were isolated from rat day 5 pups, plated on gelatin-coated plates, changed 24 hours later to the ischemic medium and cultured for 16 hours under hypoxic conditions (ischemia), or left untreated (control). Cells were fixed with PFA 4% and immunostained for cardiac-specific actinin (red), cleaved caspase-3 (green). Chromatin was stained with the Hoechst dye (blue). Insets: higher magnification of nuclear morphology. The images are representative of three independent experiments.

Fig. 3. *Immunofluorescence detection of caspase-3 activation and nuclear fragmentation in adult cardiomyocytes.* Adult cardiomyocytes were isolated from 6-month-old male rats, plated on collagen-coated plates, changed 24 hours later to the ischemic medium and cultured for 16 hours under hypoxic conditions (ischemia), or left untreated (control). Cells were fixed with PFA 4% and immunostained for cardiac-specific actinin (red), cleaved caspase-3 (green). Chromatin was stained with the Hoechst dye (blue). Insets: higher magnification of nuclear morphology. The images are representative of three independent experiments.

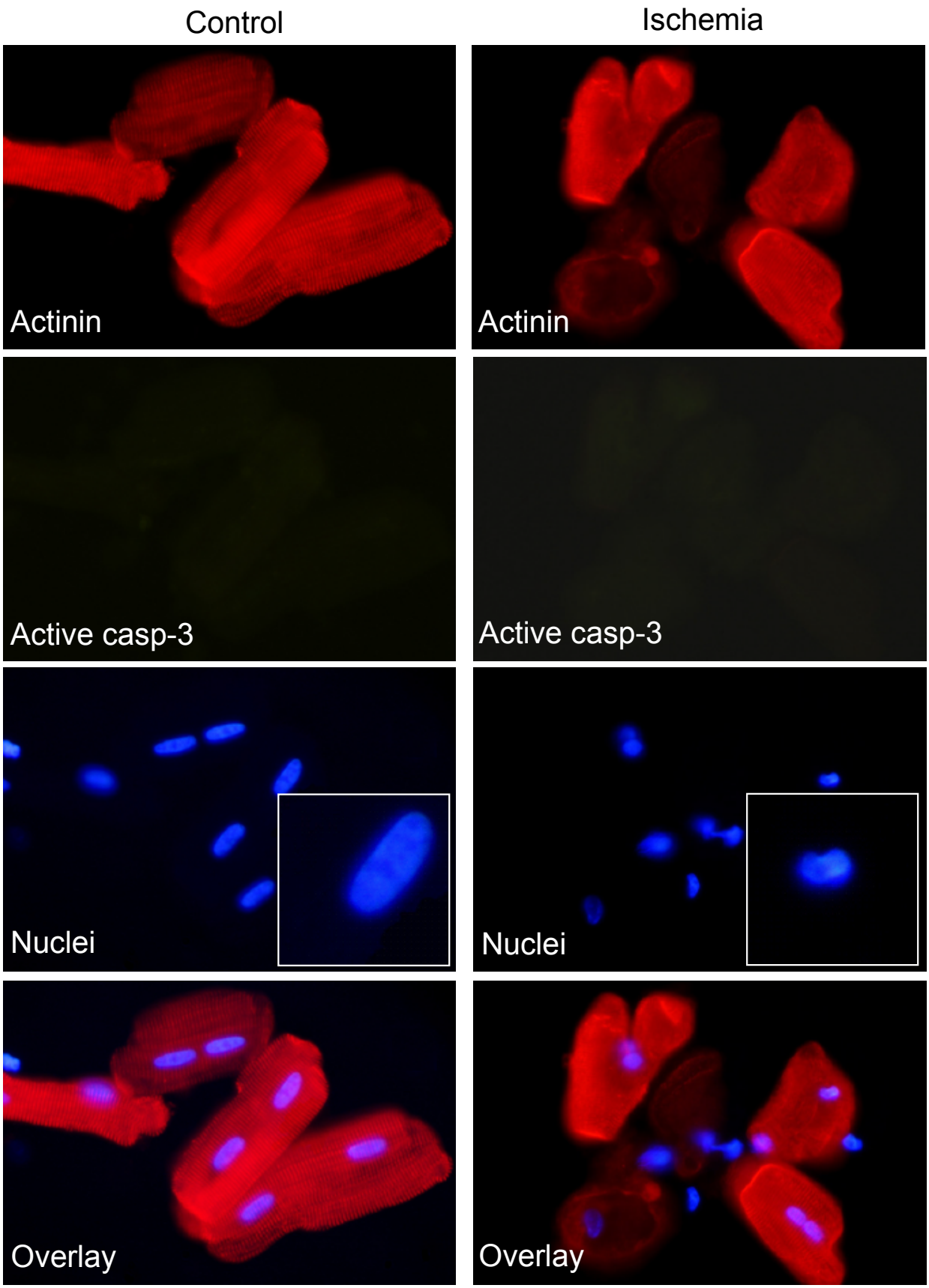
Bahi et al. Suppl. Figure 1



Bahi et al. Suppl. Figure 2



Bahi et al. Suppl. Figure 3



Results

Block 3.

EndoG is the link between BNip3-induced mitochondrial damage and TUNEL-positive, caspase-independent, DNA damage in ischemic cardiomyocytes

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EndoG is the link between BNip3-induced mitochondrial damage and TUNEL-positive, caspase-independent, DNA damage in ischemic cardiomyocytes

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ABSTRACT

Mitochondrial dysfunction and DNA damage are important steps in the cell death process. Besides the well known caspase-dependent intrinsic pathway, integrity of mitochondria and DNA can be affected without the engagement of caspases. The atypical BH3-only protein BNip3 is involved in the process leading to DNA fragmentation during ischemia. However, the pathway by which DNA degradation ensues from BNip3 activation is unknown. Our results show that TUNEL positive DNA damage in ischemic cardiomyocytes is caspase-independent and that ischemia-induced DNA high and low molecular weight fragmentation can be blocked by repressing EndoG expression in these cells. Silencing expression of BNip3, but not Nix, reduced EndoG translocation and blocked DNA degradation. Bcl-XL overexpression retained EndoG in the mitochondria and blocked DNA damage during ischemia. Thus, our results show that DNA fragmentation in differentiated cardiomyocytes during ischemia depends on EndoG activity promoted by BNip3 and prevented by Bcl-XL in a process independent of caspases that is detected by the TUNEL assay.

Keywords:

Apoptosis, BNip3, EndoG, Bcl-XL, caspase, ischemia, cardiomyocyte, DNA fragmentation

INTRODUCTION

Caspase activation has been involved in the pathological death process of neurons and cardiac myocytes. However, compelling results show that the canonical apoptotic signaling pathway is silenced in postmitotic cells during development (Yakovlev et al., 2001; Donovan and Cotter, 2002; Bahi et al., 2006). In this context, the full elucidation of the mechanisms mediating cardiomyocyte damage during ischemia could be relevant for understanding alternative pathways to caspase-dependent cell death and developing effective therapies.

Activation of the mitochondrial pathway of apoptosis in many cell types includes interaction of a BH3-only protein with the outer mitochondrial membrane (OMM), mitochondrial dysfunction, assembly of the apoptosome, caspase activation and DNA processing by Caspase-Activated DNAse (CAD) (reviewed by Danial and Korsmeyer, 2004). BNip3 and Nix form an atypical subfamily of the BH3-only proapoptotic proteins related to Bcl-2 (Chen et al., 1999). Their expression is activated by hypoxia, involving Hypoxia Inducible Factor-1 (HIF-1)-dependent transcription (Bruick 2000, Guo et al., 2001; Sowter et al., 2001). Surprisingly, BNip3 has been shown to be abundant in the adult heart (Hamacher-Brady et al. 2007). BNip3 has been involved in hypoxia/acidosis-induced DNA damage in cardiomyocytes (Kubasiak et al., 2002). Increased resistance to hypoxia-induced cell death of pancreatic and colorectal cancer cells deficient for BNip3 further supports the relevance of BNip3 during hypoxia-induced death (Okami et al., 2004; Bacon et al., 2006). Integration of BNip3 in the OMM leads to mitochondrial damage (Chen et al., 1997; Cizeau et al., 2000; Ray et al., 2000), including opening of the

mitochondrial permeability transition pore (Vande Velde et al., 2000). However, translocation of cytochrome c arising from BNip3 activity remains controversial (Vande Velde et al., 2000; Kubasiak et al., 2002). Deficiency in BNip3 in vivo has been shown to promote myocyte survival after ischemia/reperfusion (Diwan et al., 2007). Finally, BNip3 induces caspase-independent nuclear DNA degradation (Vande Velde et al., 2000; Kubasiak et al., 2002). This fact has been described as surprising (Vande Velde et al., 2000), unusual (Guo et al., 2001; Kubasiak et al., 2002), interesting (Lamy et al., 2003) and paradoxal (Webster et al., 2005), but remains unexplored.

Endonuclease-G (EndoG) is a mitochondrial protein with DNAse/RNAse activity (Gerschenson et al., 1995), which has been recently involved in apoptotic DNA degradation (Li et al., 2001; Parrish et al., 2003). EndoG apoptotic activity requires its release from mitochondria, and occurs in the absence of caspase activation (Li et al., 2001). Although EndoG activity is not evident in caspase-dependent paradigms (Irvine et al., 2005; David et al., 2006), we have recently reported that EndoG is important for DNA processing in cardiomyocytes, where the caspase-dependent pathway is repressed during differentiation and is not re-expressed during experimental ischemia (Bahi et al., 2006; Zhang et al., 2007). Therefore, EndoG was a good candidate for executing caspase-independent DNA processing in the BNip3-mediated cell death pathway. The results presented here support this hypothesis and help to clarify other functional aspects dealing with cardiomyocyte cell death during ischemia, such as the role of endogenous BNIP3 in triggering translocation of mitochondrial apoptotic regulators, and the nature of the TUNEL labeling in the ischemic myocardium.

MATERIALS AND METHODS

Cell culture and preparation of cytosolic extracts

Rat neonatal cardiomyocytes were obtained from the heart of 3-5 day-old Sprague–Dawley rats as described elsewhere (Sanchis et al., 2003). Experimental ischemia was achieved by culturing cells in Tyrode's solution (NaCl 137 mM, KCl 2.7 mM, Na2HPO4 8 mM, KH2PO4 1.5 mM, CaCl2 0.9 mM and 0.5 mM, initial pH: 7.2) inside a hypoxic chamber (Ruskin InVivo400, Cultek) continuously monitored for achieving 0.2% O_2 and 5% CO_2. Pan-caspase inhibitor Z-Val-Ala-Asp(OMe)-CH_2F (z-VAD-fmk) (Calbiochem) was added at 100μM final dilution where indicated. Cytosolic fractions were obtained as previously reported (Sanchis et al., 2003) at the end of the treatments in order to detect the release of apoptotic factors from the mitochondria.

Lentiviral vectors for gene silencing

EndoG small hairpin RNA interference (shRNA) construct has been described elsewhere (Bahi et al., 2006). The BNip3 and Nix shRNA vectors were obtained following the same protocol as for EndoG, using the sequences 5'-GATACCAACAGAGCTGAAATA-3' and 5'-GAAGACGGGCAAATAATGT-3', respectively. Each specific shRNA vector was transfected into HEK293T cells together with the plasmids psPAX2 and pMD2G (from Dr. Trono, Geneva), using the polyethyleneimine transfection method (SIGMA). Transfection efficiency was analyzed by detection of the Enhanced Green Fluorescence Protein (EGFP) expression. The final viral pellet was diluted in sterile PBS plus 2% bovine serum albumin and titrated by transduction of cardiac fibroblasts before treating

cardiomyocytes. Cardiomyocytes were transduced four hours after seeding. Efficiency of transduction was estimated to be over 90% by detection of EGFP expression at 48 hours. The maximum efficacy of the shRNA plasmids for reducing endogenous expression of the genes was determined to reach a plateau from four to six days after transduction, depending on the gene. Therefore, treatment of cardiomyocytes was started always five days after transduction, i.e. after seeding. RT-PCR primers for detecting BNIP3 were Forward: 5'-GAATCTGGACGAAGCAGCTC-3' and Reverse: 5'-AACATTTTCTGGCCGACTTG-3' with an expected amplicon size of 230 bp, and Nix primers Forward: 5'-CAATGGAGACATGGAGAAGAT-3' and Reverse 5'-ATGTTTTCGGGTCTACTGGA-3' gave an amplicon of 252bp. All experiments carried out with transduced cardiomyocytes were repeated at least four times.

Lentiviral-driven overexpression of EndoG-FLAG and Bcl-XL

For EndoG-FLAG overexpression we obtained the EndoG-FLAG fragment from the pcDNA3.EndoG-FLAG construct (Bahi et al., 2006) and subcloned it into the pEIGW vector for lentiviral transduction. The ORF of the human Bcl-XL was subcloned into pCRII and then into the pEIGW vector for lentiviral overexpression. Viral particles where obtained as previously described (Bahi et al., 2006).

Western Blot and Immunofluorescence detection

Whole cell lysates were obtained by addition of Tris-2%SDS pH: 6.8 buffered solution to the cell cultures at the end of the treatments. Protein extracts were denatured by heat shock at 95ºC for 3 minutes and quantified by the Lowry Assay (BioRad). Equal amounts of protein were electrophoresed by SDS-PAGE,

transferred to PVDF membranes (Amersham), and probed with specific antibodies. Antibodies used were against FLAG (SIGMA), BNip3 (abcam 65874), EndoG (SIGMA E5654, ProSci PSC-3035 and Santa Cruz sc-26924), Cytochrome c (Cell Signaling), Hsp60 (BD Transduction), LDH (Rockland), PTB (abcam). Because commercially available antibodies against EndoG detected unspecific bands (different size than EndoG and the signal was not reduced in cells with specific knockdown of EndoG efficiently repressed as assessed by RT-PCR), we prepared a polyclonal antibody against a peptide from the C-terminal region of EndoG (Antibody Barcelona BCN4778) that is specific and useful for western blot detection but not for immunofluorescence. Immunofluorescence was performed after fixing cells either with 4% PFA for 30 minutes or 100% methanol for 5 minutes, as previously described (Bahi et al., 2006).

DNA Integrity Assay. Cells were pelleted at the end of each treatment and frozen at -80 °C. Pellets from the same experiment were processed at once. They were diluted in 40 µl of sterile phosphate-buffered saline, mixed with 40 µl of melted 1% low melting agarose (Sigma) in 0.5x TBE (45 mM Tris pH 8.3, 45 mM boric acid, 1.0 mM EDTA; all reagents from SIGMA). Each mixture was poured into a block caster and let solidify. Each agarose block was submerged into 1 ml of lysis buffer (1% lauryl sarcosil, 0.5 M EDTA, 10 mM Tris, pH 8, 100 µg/ml proteinase K; all reagents from SIGMA) at 50°C during 24 h in mild agitation, and rinsed twice with 0.5x TBE for 1 h at room temperature. For analysis of DNA high molecular weight degradation, the blocks were then laid into wells of a 1% agarose, 0.5x TBE gel (CHEF grade, SIGMA-Aldrich). Pulse field electrophoresis was performed as previously described (Bahi et al., 2006) in a CHEF DR-II system (Bio-Rad,

Hercules, CA, USA) set to the following protocol: run time, 14 h; switch time from 5 to 50 s; voltage gradient, 6 V/cm. Gels were stained with SYBR Safe (Molecular Probes-Invitrogen), visualized by UV exposure and recorded with a Kodak DC290 digital camera. Densitometry was performed as described above.

RESULTS

BNip3 and EndoG trigger caspase-independent, TUNEL positive DNA fragmentation in ischemic cardiomyocytes.

BNip3 has been involved in chromatin condensation (Chen et al., 1997), and in ischemia-induced cardiomyocyte DNA low molecular weight (LMW) fragmentation (Kubasiak et al., 2002). Although the BNip3-related protein Nix also has a pro-apoptotic role (Chen et al., 1999) and its expression is up-regulated during hypoxia in several cell lines (Bruick et al., 2000; Sowter et al., 2001), its involvement in hypoxia-induced cell death is less clear (Galvez et al., 2006). We have developed a lentiviral-based small hairpin RNA interference system (shRNA) for silencing efficiently BNip3 and Nix expression in primary postnatal cardiomyocytes (Fig.1A). Silencing BNip3 expression, but not that of Nix, blunted high molecular weight (HMW) and LMW degradation of DNA during experimental ischemia (Fig.1B). The effect of BNip3 silencing on DNA integrity during ischemia was similar to this of repressing EndoG (Fig.1C). Both EndoG and BNip3, but not Nix, were essential for inducing TUNEL-positive DNA fragmentation (Fig.1D). These data broaden former knowledge by showing that BNip3, but not Nix, is involved in ischemia-induced DNA high as well as low molecular weight processing, and that ischemic DNA damage detected by the TUNEL assay proceeds in the absence of caspase activation in cardiomyocytes.

BNip3 controls EndoG release from cardiac mitochondria during ischemia.

Experimental ischemia induced the release of EndoG-FLAG from cardiomyocyte mitochondria and endogenous EndoG is involved in ischemia-induced, caspase-

independent DNA degradation in these cells (Bahi et al., 2006). Our results showed that BNip3 is involved in the DNA fragmentation induced by ischemia in agreement with Kubasiak et. al. 2002. Thus, we analyzed the translocation of EndoG with the treatment of BNip3 RNA interference in neonatal cardiomyocytes to verify whether BNip3 induces DNA damage by controlling the release of EndoG. Neonatal cardiomyocytes overexpressing EndoG-Flag were transduced with scrambled control or BNip3 RNA interference constructs. At day 4 after transduction cardiomyocytes were treated with 12 hour ischemia, and then were fixed with 4% paraformaldehyde and immunostained for Flag. Our results show that in ischemia treated cardiomyocytes EndoG-Flag signal has a diffused pattern including nucleus, whereas BNip3-interference treated cell showed a pattern similar with control cell (Figure 2), suggesting that BNip3 can trigger the release of EndoG from mitochondria during ischemia.

Bcl-XL can block EndoG translocation and caspase-independent DNA damage in ischemic cardiomyocytes.

Our results show that silencing of endogenous EndoG expression by shRNA blocked ischemia-induced HMW and LMW fragmentation of DNA and the nuclear TUNEL labeling (Fig.1). Thus, EndoG was involved in the production of DNA -3'OH single strand nicks detected by the TUNEL assay, which has been routinely used as a measure of Caspase-Associated DNAse (CAD) activity and, hence, of caspase activation in cardiomyocytes, and that BNip3 triggers EndoG translocation (Fig.2). These results demonstrate that EndoG is the essential downstream executor of BNip3-induced DNA damage. In order to know whether EndoG translocation during ischemia in cardiomyocytes can be prevented, we induced lentiviral-driven

overexpression of Bcl-XL in cultured cardiomyocytes before ischemia. We analyzed EndoG expression in by immunofluorescence in EndoG-FLAG overexpressing cardiomyocytes cultured in normal conditions and cardiomyocytes treated with ischemia of six hours. Paraformaldehyde fixation allowed detection of EndoG-FLAG even when released from mitochondria (Fig.3 B). In these conditions, the pattern of EndoG-FLAG expression was diffuse during ischemia (arrows), suggesting translocation, however paraformaldehyde fixation did not allow detection of a mitochondrial marker (data not shown). To complete these results, we fixed other plates from the same experiment with methanol. This procedure allowed co-staining of EndoG-FLAG and the mitochondrial marker Hsp60, demonstrating co-localization (merged image) in control conditions. However, in ischemic cardiomyocytes, the FLAG staining was lost in absence of Bcl-XL overexpression, probably by diffusion from the cytosol outside the cells during the procedure. These complementary results suggest that EndoG-FLAG is released from mitochondria during ischemia and that Bcl-XL simultaneous overexpression can block this event. Finally, we assessed where Bcl-XL was efficient in blocking EndoG nuclease activity. DNA integrity was analyzed in simultaneous experiments by CHEF agarose gel electrophoresis (Fig.3 D). The results show that Bcl-XL overexpression blocks caspase-independent DNA high molecular weight fragmentation during ischemia, at least for 6 hours of ischemia. Our results showed that Bcl-XL efficiently blocks translocation of overexpressed EndoG and prevented DNA damage in ischemic cardiomyocytes.

DISCUSSION

The events contributing to ischemia-induced heart disease have been elucidated in recent years. Solid evidences demonstrate the important role of cardiomyocyte cell damage in heart failure (reviewed by Foo et al., 2005). However, in our opinion, confusion still remains about the intracellular mechanisms involved in cardiomyocyte damage during ischemia and ischemia/reperfusion. Here, we show that ischemia-induced TUNEL-positive DNA damage in ischemic cardiomyocytes in vitro is triggered by EndoG and depends on EndoG release from mitochondria induced by BNip3 in a way that can be blocked by Bcl-XL, without the involvement of caspases.

The contribution of the canonical caspase-dependent apoptotic pathway to ischemia-induced cardiomyocyte cell death was initially described by Tanaka et al., 1994. The extrinsic, death receptor-dependent, apoptotic cascade (Lee et al., 2002 and many others) and the mitochondria-dependent pathway of apoptosis (reviewed by Crow et al., 2004) have been suggested to contribute to cardiomyocyte death during ischemia or ischemia/reperfusion. However, simultaneous reports showed that myocyte damage and death were unrelated to caspases (Knaapen et al., 2001; Chen et al., 2002; Kubasiak et al., 2002; Bahi et al., 2006). The lack of caspase activity in ischemic cardiomyocytes is in agreement with our previous finding that expression of the apoptotic genes is silenced during cardiomyocyte differentiation (Bahi et al., 2006; Zhang et al. 2007). Using different approaches, it has been shown that Bcl-2 and Bcl-XL could blunt ischemia-induced cardiomyocyte damage (Chen et al., 2001; Chattergee et al., 2002; Huang et al., 2003), also suggesting the involvement of the caspase-dependent pathway triggered by cytochrome c translocation. The use of the

TUNEL assay and DNA electrophoresis to assess DNA fragmentation as reliable methods for detecting apoptotic myocytes in human samples contributed to the knowledge of the role of cell damage in heart failure but biased the interpretation of the mechanisms involved in this event (Narula et al., 1996; Saraste et al. 1997; Olivetti et al., 1997; Veinot et al., 1997).

Although many excellent works have unveiled the essential role of BNip3 in DNA damage in myocytes and other cell types (Vande Velde et al., 2000; Guo et al., 2001; Kubasiak et al., 2002; Lamy et al., 2003), the nuclease executing BNip3-dependent DNA damage has not been identified so far. Here we show that endogenous BNip3 induces EndoG release and caspase-independent EndoG-dependent DNA fragmentation, supporting a role of EndoG as the executioner of DNA damage triggered by BNip3. We also extent the knowledge about the role of BNip3 in DNA damage by showing for the first time that high molecular weight DNA fragmentation also depends on BNip3 activity in ischemic cardiomyocytes. In addition, although cell damage induced by BNip3 depend on Bax and Bak activation and induce caspase activation in caspase-dependent cellular models such as embryonic fibroblasts (MEF) and the immortalized embryonic atrial myocytes (HL-1) (see review in Burton and Gibson, 2009), our results give support to a role of BNip3 unrelated to caspases in differentiated cardiomyocytes as previously shown by others (Kubasiak et al., 2002) and also suggest a caspase-independent mechanism for the known protective role of overexpressed Bcl-XL in the heart in vivo (Huang et al., 2003).

The link between TUNEL-positive DNA fragmentation and caspase activity, which is true for other cellular models and that has been assumed for cardiomyocytes, was questioned by investigations performed in tissue samples (Knaapen et al., 2001; French et al., 2009). Our data demonstrate that the TUNEL signal is produced by the activity of EndoG without the contribution of caspases in ischemic cardiomyocytes.

In summary, our results provide evidence that EndoG is the link between BNip3-mediated mitochondrial dysfunction and DNA degradation during ischemia and that this pathway is unrelated to caspase activation in cardiomyocytes. In addition, we show that TUNEL labeling of ischemic cardiomyocytes, which has been used frequently as a marker of caspase-dependent apoptosis, is due to EndoG and is unrelated to caspase activity in these cells. Finally, our results show that Bcl-XL can prevent ischemia-induced DNA fragmentation by blocking EndoG translocation from mitochondria. Thus, the evidence that BNip3 and EndoG form a pathway for large-scale and low molecular weight DNA degradation in cardiomyocytes unrelated to caspase activation add information to previous reports supporting caspase-independent mechanisms during cardiac ischemia and ischemia-reperfusion, clarify the induction of caspase-independent DNA damage by BNip3, demonstrate that the TUNEL assay detects caspase-independent, EndoG-dependent DNA damage in ischemic myocytes and agree with a type of cell damage unrelated to caspases that can take place after the global expression silencing of genes of the caspase-dependent signaling cascade during myocyte differentiation (Bahi et al., 2006; Zhang et al., 2007; Doss et al., 2007).

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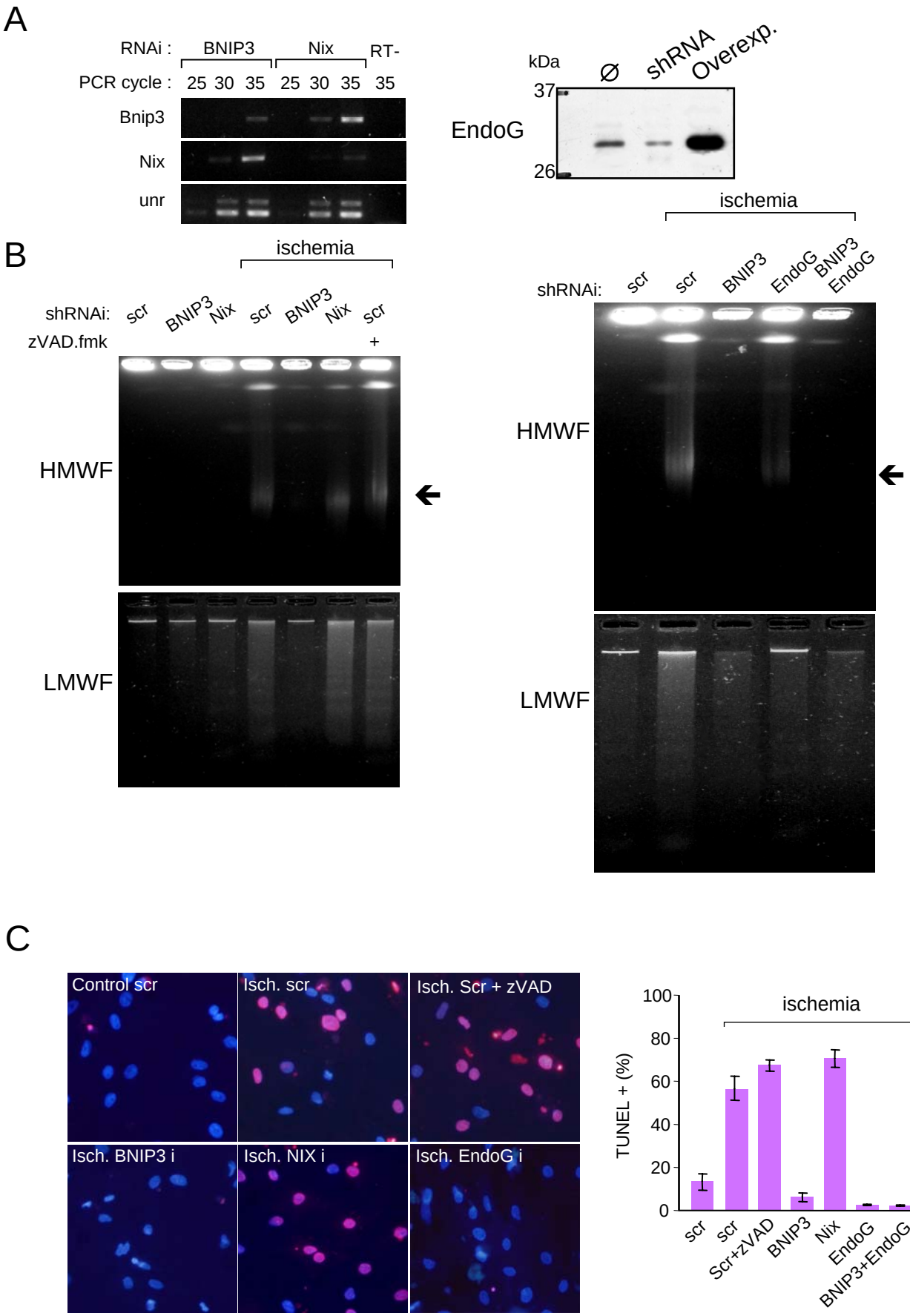
FIGURE LEGENDS

Figure 1. BNip3 and EndoG trigger caspase-independent, TUNEL positive DNA fragmentation in ischemic cardiomyocytes. A) Efficiency of Nix, BNip3 and EndoG shRNA-mediated knock down was analyzed by RT-PCR or western blot from postnatal cardiomyocyte total RNA or protein extracted at day 4 post-transduction. B) Agarose gel electrophoresis of DNA extracts of cardiomyocytes cultured in normal conditions or after 16 hours of experimental ischemia. Cardiomyocytes were transduced 5 days before ischemia with constructs for the silencing of BNip3, Nix or EndoG genes. A sample from scrambled-transduced cardiomyocytes submitted to ischemia in the presence of 100μM pan-caspase inhibitor zVAD.fmk was added to show that DNA fragmentation was caspase-independent in this paradigm. HMWF: High molecular weight DNA fragmentation detected as reported in the Materials and Methods section. LMWF: Low molecular weight DNA fragmentation detected by conventional agarose gel electrophoresis from the same cell extracts. C) DNA integrity was measured following the same treatments as in B by the TUNEL assay. Gel images are representative of three independent experiments. TUNEL-positive cells are expressed as mean vs. total nuclei from three independent experiments counted in duplicates. Error bars are SEM.

Figure 2. BNip3 controls EndoG release from cardiac mitochondria during ischemia. Immunofluorescence of EndoG-FLAG in overexpressing neonatal cardiomyocytes cultured in control (control) and ischemic conditions for 12 hours (ischemia). BNIP3i: cardiomyocytes with silenced expression of BNIP3 by shRNA.

Similar results were obtained in three independent experiments. Arrows: cardiomyocytes where EndoG-FLAG expression does not exclude de nucleus.

Figure 3. Bcl-XL can block EndoG translocation and caspase-independent DNA damage in ischemic cardiomyocytes. A) Assessing overexpression of Bcl-XL in normal cardiomyocytes and simultaneous EndoG-FLAG overexpression. B) Immunofluorescence of EndoG-FLAG in control and ischemic (6 hours) cardiomyocytes transduced previously with viruses inducing EndoG-FLAG overexpression and empty viral particles (∅) or particles inducing Bcl-XL overexpression. Cells were fixed with paraformaldehyde. This procedure allows detection of EndoG-FLAG, even when it is released from mitochondria, but not the mitochondrial marker Hsp60 (see the results section). C) Immunofluorescence of EndoG-FLAG in control and ischemic (6 hours) cardiomyocytes transduced previously with viruses inducing EndoG-FLAG overexpression and empty viral particles (∅) or particles inducing Bcl-XL overexpression. Cells were fixed with methanol. This procedure does not allow EndoG-FLAG detection when released from mitochondria but allows simultaneous detection of the mitochondrial marker Hsp60. D) Agarose gel electrophoresis of DNA extracts of cardiomyocytes cultured in normal conditions or after 6 hours of experimental ischemia. Cardiomyocytes were transduced 5 days before ischemia with lentivirus for overexpression of Bcl-XL or empty viruses. HMWF: high and low molecular weight DNA fragmentation. Similar results were obtained in three independent assays.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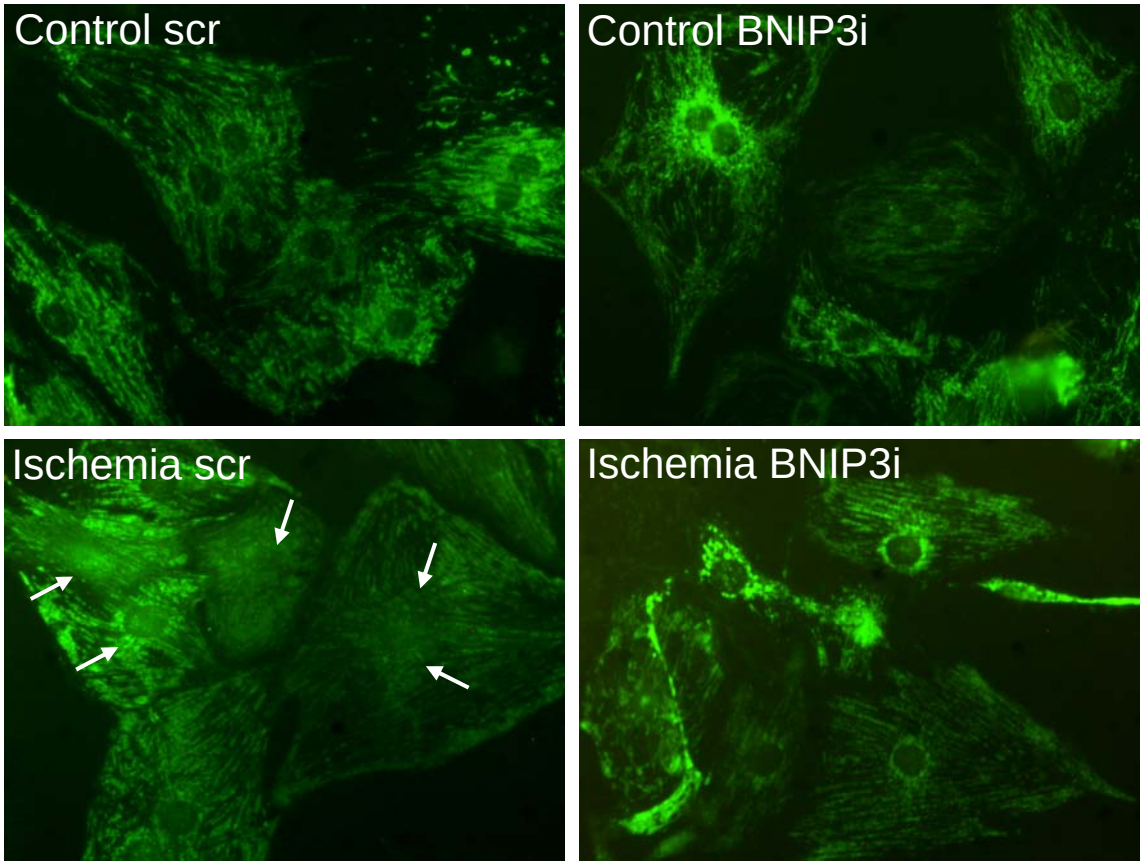
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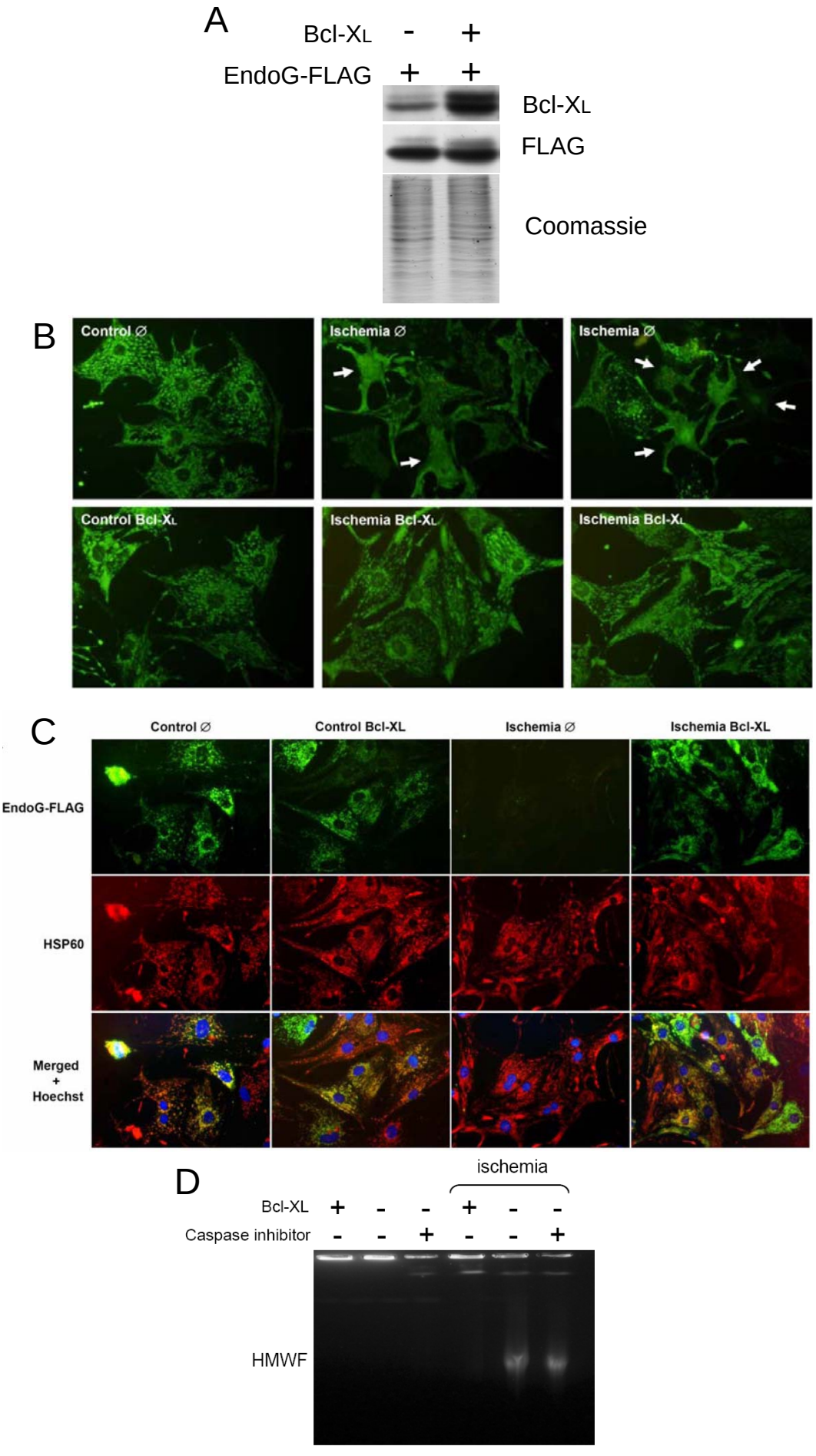
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Results

Block 4.

Polypyrimidine tract binding proteins (PTB) regulate the expression of apoptotic genes and susceptibility to caspase-dependent apoptosis in differentiating cardiomyocytes.

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Polypyrimidine tract binding proteins (PTB) regulate the expression of apoptotic genes and susceptibility to caspase-dependent apoptosis in differentiating cardiomyocytes

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Cardiac morphologic abnormalities in mice deficient for key regulators of the caspase-dependent signaling underscored its role in heart development. However, the mechanisms regulating apoptotic gene expression in the developing heart are unknown. As polypyrimidine tract binding proteins (PTB) determine gene isoform expression during myoblast differentiation and contribute to Apaf-1 translation in cell lines, we investigated whether PTB regulate apoptotic gene expression in differentiating cardiomyocytes. Our results show that PTB are expressed in the embryonic heart and are silenced during development, coinciding with a reduction in the expression of apoptotic genes. Overexpression of PTB in postnatal cardiomyocytes, which express low levels of PTB and apoptotic genes, induced an increase in the amount of pro-apoptotic proteins without affecting abundance of their respective transcripts. Translation of the reporter gene Firefly Luciferase preceded by the 5′-untranslated region of Apaf-1 or Caspase-3 was enhanced by PTB in cardiomyocytes. PTB silencing in fibroblasts induced a decrease of apoptotic protein levels. PTB overexpression in cardiomyocytes induced caspase activity and caspase-dependent DNA fragmentation during ischemia, which is otherwise caspase-independent in differentiated cardiomyocytes. Our results show that PTB contribute to apoptotic gene expression and modulate the susceptibility to caspase activation in differentiating rat cardiomyocytes.
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Apoptotic signaling involves an intracellular proteolytic cascade triggered by environmental stimuli through membrane death receptors, or by internal inputs detected by mitochondria and the endoplasmic reticulum.[1] In addition to contribute to cell death, some regulators of the apoptotic signaling cascade can also influence cell differentiation.[2] Increasing evidence shows that apoptotic signaling contributes to heart organogenesis.[3–5] Blockade of caspase activity with several caspase inhibitors induced alterations in the ventriculoarterial connections in the chick embryo,[4] whereas deletion of genes coding for upstream regulators of apoptosis such as FADD and cFLIP as well as for executioner caspases in the mouse induced ventricular wall noncompaction and lethality.[3,5,6] Thus, abnormal expression of apoptotic genes could be involved in some inherited and congenital heart diseases, although many of the underlying mechanisms remain unknown.

Our earlier findings suggested that the apoptotic machinery is expressed in the myocardium during embryonic development and is silenced after birth in different rodent models.[7,8] Apoptotic gene silencing has been also found in stem cells after cardiac-directed differentiation *in vitro*.[9] We hypothesized that apoptotic gene silencing in cardiomyocytes is associated with their differentiation and or exit of cell cycle, and we analyzed the expression in the developing heart of several proteins known to regulate apoptotic gene expression, cell cycle progression, and differentiation in other cellular models. We reported earlier that expression of the cell cycle regulators E2F parallels the pattern of apoptotic gene expression in the developing heart.[8] However, E2F deficiency does not affect apoptotic gene expression in the heart *in vivo*[8] despite inducing Apaf-1 expression in cell lines.[10]

Polypyrimidine tract binding proteins 1 and 4 (PTB) are RNA-interacting proteins coded by the same gene that have important functions in RNA maturation and protein translation.[11,12] Binding to precise pyrimidine-rich motifs in pre-mRNAs, PTB1, and PTB4 proteins induce skipping of the adjacent exon determining isoform gene expression or premature termination of the open reading frame.[13,14] In addition, PTB contribute to internal ribosome entry segment (IRES)-dependent protein translation,[12] including trranslation of the pro-apoptotic adaptor Apaf-1.[12,15] Recently, PTB expression has been shown to be repressed during differentiation in neurons[16,17] and in C2C12 myoblasts *in vitro*,[18]

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Keywords: apoptosis; differentiation; cardiomyocyte; gene expression
Abbreviations: PTB, polypyrimidine tract binding proteins; nPTB, endogenous neural PTB; RRMs, RNA recognition motifs
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influencing gene isoform expression. Here, we show that expression of PTB is abundant in the developing rat heart and is progressively repressed after birth, and we characterize the role of PTB in apoptotic gene expression and susceptibility to caspase-dependent apoptosis in differentiating cardiomyocytes.

Results

Apoptotic proteins are abundant in embryonic rat cardiomyocytes but are scarcely expressed in adult cardiomyocytes (Figure 1a) as reported earlier.[8] We found that polypyrimidine tract binding proteins PTB1 and PTB4, which control gene expression through mRNA maturation and translation, are abundant in the developing heart but disappear progressively after birth both in rat and mouse (Figure 1b). This fact was associated with the differentiation process of cardiomyocytes as suggested by the inverse correlation between the expression pattern of PTB and cardiac α-actin in the myocardium (Figure 1b, bar graph). PTB expression silencing during heart development implied the progressive reduction of the level of their transcripts (Figure 1c).

We had shown earlier that although embryonic cardiomyocytes express high levels of apoptotic genes and readily activate caspases in stress situations, postnatal and adult rat cardiomyocytes express low levels of apoptotic genes and die by caspase-independent mechanisms.[7] We hypothesized that PTB is involved in apoptotic gene expression in the developing heart and that PTB silencing could contribute to decrease apoptotic gene expression in postnatal myocites. Lentiviral-driven overexpression of the human sequences of PTB1 and PTB4 in P4-5 postnatal cardiomyocytes, which express low levels of PTB and apoptotic genes, induced an increase of apoptotic protein expression (Figure 2). However, PTB expression did not alter the steady-state amount of apoptotic gene transcrips (Figure 3 and data not shown). As expected, expression of either human PTB1 or PTB4 induced a slight decrease in endogenous PTB transcripts (Figure 3, second row from top) and skipping of their target exon 10 in the transcript of endogenous neural PTB (nPTB)[19] (Figure 3).

In an attempt to identify the posttranscriptional mechanisms involved in PTB-dependent apoptotic gene expression in cardiomyocytes, we performed Luciferase reporter gene assays using bicistronic plasmids in which stabilized Firefly Luciferase (LucF $+$) is placed after the 5′-untranslated region (5′-UTR) of Apaf-1 or Caspase-3. In this system, *Renilla* Luciferase (LucR) is located at the 5′-end of the bicistronic transcript, it is translated by the cap-dependent machinery

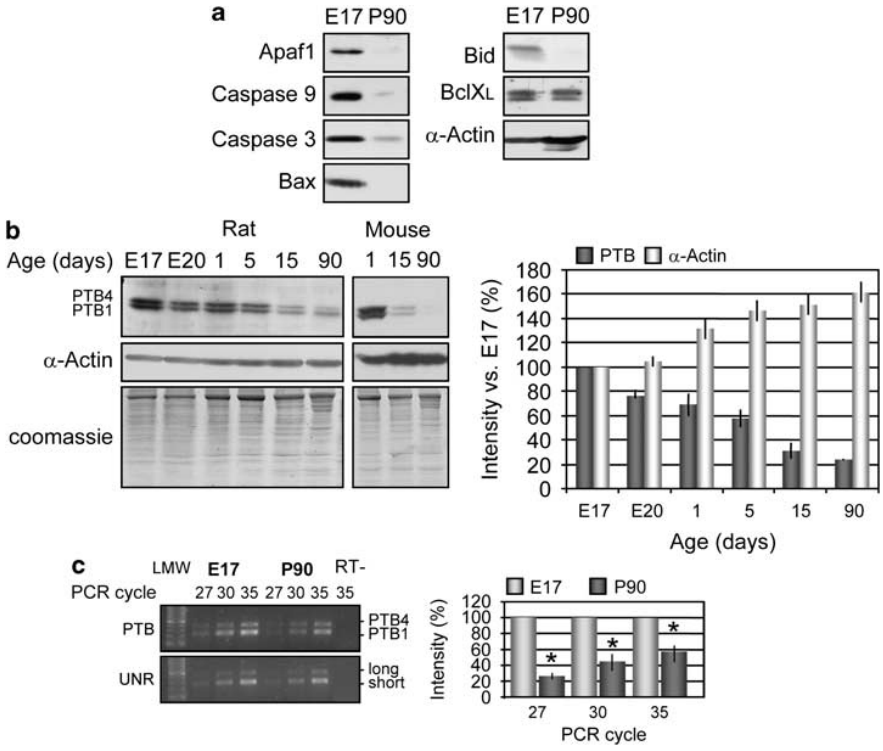

Figure 1 Expression of PTB1 and PTB4 is spontaneously reduced during cardiac development *in vivo* in parallel to apoptotic gene silencing. (**a**) Expression of apoptotic proteins in ventricles of embryonic (E17) and adult (P90) rats. (**b**) Expression of PTB1 and PTB4 proteins in ventricles of rats and mice of different ages from embryonic day 17 (E17) to adult P90. Bar graph: densitometric quantification of western blot signals of PTB and α-actin protein expression in rat ventricles ($n = 3$, error bars: S.E.M.). Values are referred to the signal at embryonic day 17. (**c**) Semi-quantitative analysis of PTB1 and PTB4 transcripts in embryonic day 17 (E17) and adult (P90) ventricles. The gel image shows samples obtained at PCR cycles 27, 30, and 35 for PTB and UNR. Densitometric analysis of the sum of PTB 1 and 4 bands normalized to E17 values is shown for each PCR cycle; $*P < 0.001$ *versus* E17 signal at the same PCR cycle. In the RT- sample, the reverse transcription reaction was omitted. UNR (upstream of n-Ras, Cold shock domain-containing protein E1 (CSDE1)) transcript was amplified as loading control as described earlier[7]

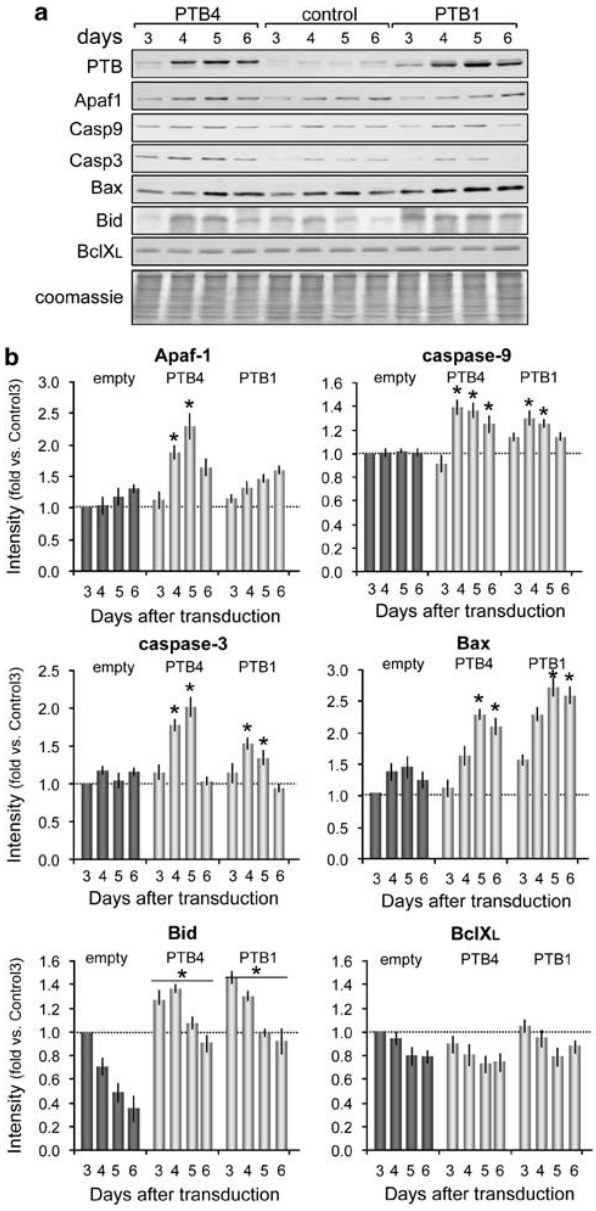

Figure 2 Overexpression of PTB1 or PTB4 in cultured neonatal cardiomyocytes induces an increase of apoptotic protein expression. (**a**) Analysis of expression of PTB and apoptotic proteins in P4-5 postnatal rat cardiomyocytes transduced with empty lentiviruses (control) or viral particles inducing expression of human PTB1 or PTB4 at days 3 to 6 after transduction. (**b**) Densitometric analysis of the signals from three to four independent experiments is expressed as fold change *versus* controls, at day 3 after transduction ± S.E.M., $*P \leqslant 0.05$ *versus* empty vector at the same day

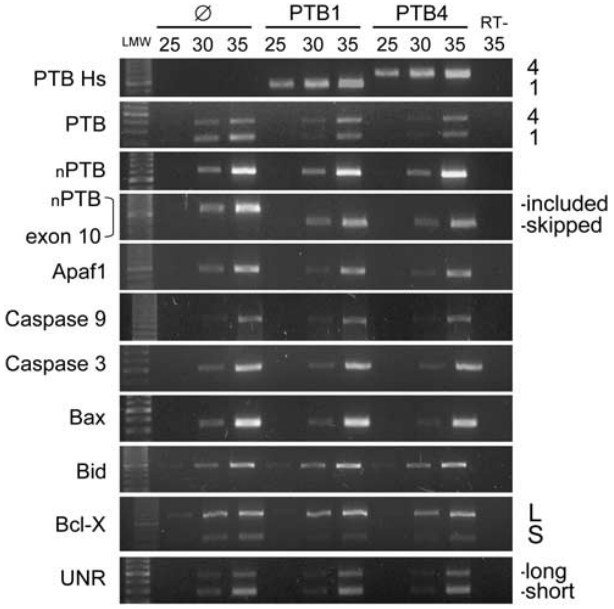

Figure 3 Overexpression of PTB1 or PTB4 in cultured neonatal cardiomyocytes does not affect the level of apoptotic gene transcripts. Steady-state transcript amounts were reverse-transcribed from total RNA extracts of postnatal cardiomyocytes transduced for 4 days with empty (∅), PTB1 or PTB4 vectors, and the cDNAs were PCR-amplified in 50 μl reaction volumes. A measure of 10 μl were sampled at PCR cycles 25, 30, and 35 and electrophoresed in agarose gels (see Materials and Methods and ref. [7]). We used primers specific for human (Hs) and rat PTB 1 and 4, primers detecting the long and short UNR transcripts, as well as primers sensitive to the inclusion/exclusion of nPTB exon 10 (see details in Supplementary Table 1). LMW: ladder of molecular weights; RT- sample: the reverse transcription reaction was omitted (cycle 35 is shown). UNR transcript was analyzed as loading control (ref.[7]). Similar results were obtained in three independent experiments

and it is used as a control of transcription/translation of the bicistronic mRNA. This system has been used for studying IRES-dependent translation in cell lines.[12,15] We transfected the bicistronic plasmids in postnatal cardiomyocytes expressing normal–low levels of PTB and cardiomyocytes overexpressing PTB, and analyzed *Renilla* and Firefly Luciferase activities 4 days later (see Materials and Methods). High PTB expression in cardiomyocytes induced an increase of LucF + activity controlled by the 5′-UTR of Apaf-1 or Caspase-3, when normalized to the values obtained in cardiomyocytes transfected with a bicistronic plasmid without any 5′-UTR (Figure 4). LucR activity was similar in all the cell extracts, confirming similar transfection rates and comparable transcription and cap-dependent translation activities. These results were in agreement with the induction of translation of apoptotic genes by PTB. Taken together, the above results show that PTB can induce apoptotic protein expression in differentiating cardiomyocytes without affecting the transcript levels of these genes, and our reporter gene experiments suggest that IRES-dependent translation could be involved in this event.

In addition to mediate IRES-dependent translation, PTB interacts with intronic regions of pre-mRNAs in the nucleus through four RNA recognition motifs (RRMs). To investigate whether the nuclear pool of PTB contributed to apoptotic gene expression, we prepared PTB mutants lacking one or more basic residues in the N-terminus that had been identified as important for nuclear localization using PTB-CAT fusion expression assays.[20] However, these mutations did not prevent accumulation of PTB in the nucleus of cardiomyocytes (Supplementary Figure 1). Then, we

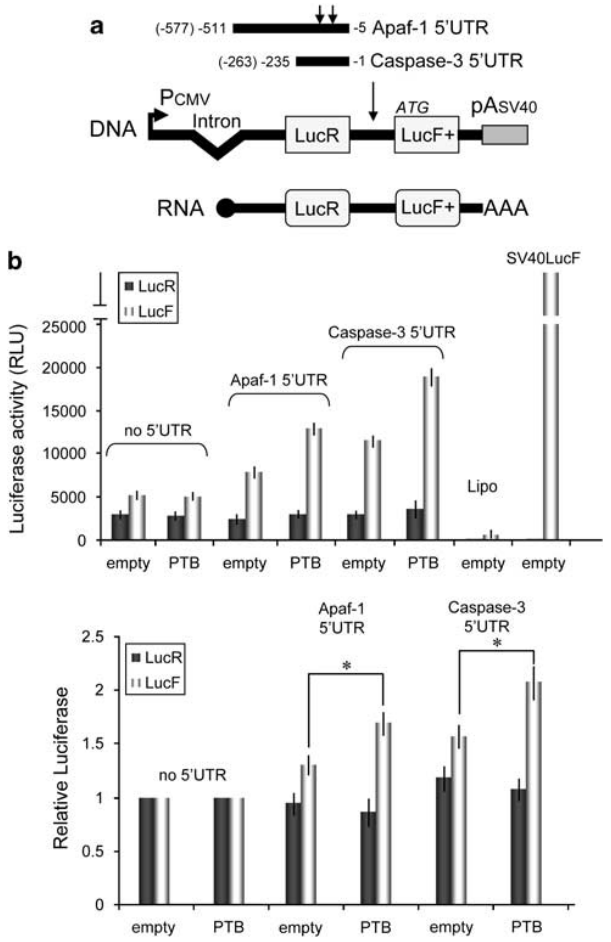

Figure 4 The 5′-untranslated region (5′-UTR) of the transcripts of Apaf-1 and Caspase-3 drive IRES-dependent translation mediated by PTB in cardiomyocytes. (**a**) Scheme of the bicistronic reporter constructs. Numbers indicate the distance from the ATG (+1) of the cloned fragments and numbers within parentheses correspond to the 5′ uppermost nucleotide found in the GenBank entry for each transcript (see Materials and Methods). Arrows in the Apaf-1 5′-UTR indicate location of two known PTB binding motifs.[15] LucR: *Renilla* Luciferase; LucF+: stabilized Firefly Luciferase. Transcription is driven by the Cytomegalovirus promoter (P_{CMV}) and finishes with the poly-A tail from the SV40 transcript (pA_{SV40}). (**b**) Luciferase activity was measured in cardiomyocytes transduced with empty viruses (empty) or viral particles inducing PTB expression and transfected with the bicistronic plasmids carrying the reporter LucF+ gene downstream of the 5′-UTR of Apaf-1 or Caspase-3. pSV40LucF, carrying the LucF gene downstream of the SV40 promoter, was used as a positive control for LucF activity and negative control for LucR activity; pCRHL, which contains a small hairpin sequence between LucR and LucF+ was used as positive control for LucR and negative control for LucF. Cardiomyocytes treated with Lipofectamine alone (Lipo) were used as background control. LucR activity allows estimating transcription of the bicistronic construct and cap-dependent translation and was used to confirm similar transfection and transcription rates. LucF+ activity takes place only if the cloned 5′-UTR drives IRES-dependent translation. Upper graph: representative experiment showing Luc activity of duplicate samples (RLU: relative luciferase units). Lower graph: Values are expressed as mean ± S.E.M. Luciferase activity normalized to pCRHL values from four experiments. $*P \leqslant 0.05$

prepared and transduced a short form of both PTB1 and PTB4 starting at Met175. Short PTB lacked the N-terminal nuclear localization signal (NLS) and the first RRM, which has been shown to influence nuclear shuttling[21,22] and to be dispensable for RNA binding.[21] Unexpectedly, although PTB short forms localized to the cytosol, an important pool was still directed to the nucleus (Figure 5a). PTB short forms retained the capacity to induce nPTB exon 10 skipping (Figure 5b) and to enhance expression of Apaf-1 and Caspase-3 but were not efficient to sustain Caspase-9, Bax, and Bid expression (Figure 5c). Although these results cannot determine whether nuclear PTB contribute to the expression of apoptotic genes, they suggest that the NLS and/or the first RRM are required to sustain Caspase-9, Bax, and Bid expression yet are dispensable for expression of other apoptotic genes. Furthermore, our results show that there are other factors in addition to the NLS and RRM1 contributing to the nuclear localization of PTB, which could have been missed in studies using fusion proteins and cell lines, as discussed below.

To investigate whether forced reduction of PTB expression would weaken apoptotic gene expression, we initially attempted to repress PTB expression through lentiviral-driven shRNA in embryonic E17 cardiomyocytes cultured as reported earlier,[7] which express high levels of PTB and apoptotic genes. However, expression of apoptotic regulators and other genes were spontaneously reduced in these cultures before the period required for achieving efficient silencing in neonatal cells (i.e. 4–5 days), precluding further insight in these cells (data not shown). Then, we repressed PTB expression with two independent PTB-specific shRNA constructs in Rat1 fibroblasts, which express high levels of PTB and apoptotic genes, compared with neonatal cardiomyocytes. Decreased PTB expression down to the 20% induced a slight yet consistent decrease in pro-apoptotic protein amounts (Figure 6), confirming that apoptotic gene expression can be modified by altering endogenous PTB activity.

To ascertain the biological relevance of the global increase of apoptotic gene expression mediated by PTB in cardiomyocytes, we treated postnatal rat cardiomyocytes transduced with empty viruses, PTB1 or PTB4 to experimental ischemia and measured executioner caspase activity, caspase-3 activation, and DNA integrity. Executioner caspase activation during ischemia in PTB1- and PTB4-overexpressing cardiomyocytes was significantly induced, contrary to wild-type cells both measured through enzymatic analysis and immunofluorescence (Figure 7). DNA damage was caspase-independent in cardiomyocytes transduced with empty vector, as reported earlier.[7] However, ischemia-induced caspase activation in PTB-overexpressing cells correlated with the induction of caspase-dependent, Z-VAD-inhibitable, high molecular weight DNA fragmentation (Figure 8). These results confirmed that global activation of apoptotic gene expression mediated by PTB in differentiating cardiomyocytes contributed to their susceptibility to the induction of caspase-dependent apoptosis.

Discussion

We have shown here that PTB1 and PTB4 are expressed in the murine myocardium during embryonic cardiac development and are down-regulated progressively after birth, after a temporal pattern similar to that of apoptotic gene expression.

Restoring PTB expression in postnatal cardiomyocytes was sufficient to increase apoptotic protein expression, probably involving IRES-dependent translation, and to induce a shift from caspase-independent to caspase-dependent DNA damage during experimental ischemia, which is caspase-independent in wild-type postnatal cardiomyocytes. To our knowledge, this is the first report on the mechanisms of regulation of apoptotic gene expression in the developing heart.

Participation of the apoptotic signaling in heart development[3,5,6] and silencing of this pathway during cardiomyocyte terminal differentiation *in vivo*[7,8] and *in vitro*,[9] suggest its strict regulation during development in the myocardium. However, the mechanisms regulating apoptotic gene expression during cardiac development are unknown. Indeed, although gain of function or inactivation of many genes have been reported to induce alteration of the normal rate of cardiomyocyte apoptosis in the developing heart, the possible involvement of changes in the expression of apoptotic genes in these phenotypes was not assessed.

Despite the involvement of PTB proteins in alternative splicing of several genes relevant for cardiomyocyte function such as α-actinin,[23] α-Tropomyosin,[24] β-Tropomyosin,[25] and Troponin-T,[26] as well as the role of PTB in the control of IRES-dependent translation of several genes including pro-apoptotic Apaf1,[15] there is no information about PTB expression and activity neither in mammalian cardiomyocytes nor in heart development. PTB has been suggested to be abundant in the adult chicken heart,[27] yet our results show that both rat and mouse myocardium spontaneously silence PTB expression early after birth.

Our observation that PTB1 and PTB4 are abundant in the developing embryonic heart and are silenced postnatally suggested to us their role in the control of gene expression during cardiomyocyte differentiation, in a similar way to what has been described in neurons[16,17] and in differentiating C2C12 myoblasts *in vitro*.[18] PTB gain-of-function experiments presented here strongly suggest that PTB contribute to the posttranscriptional regulation of apoptotic gene expression and, hence, susceptibility to apoptosis in differentiating cardiomyocytes. Interestingly, the expression of the anti-apoptotic gene Bcl-XL, unaffected by cardiomyocyte differentiation (Figure 1 and ref.[8]), was not modified by PTB. We wanted to identify the mechanisms mediating apoptotic gene expression driven by PTB in cardiomyocytes. PTB has been shown to contribute to Apaf-1 IRES-mediated translation in cell lines.[12,15] To show this, authors used bicistronic vectors in which the *Renilla* Firefly (LucR) ORF and the Firefly

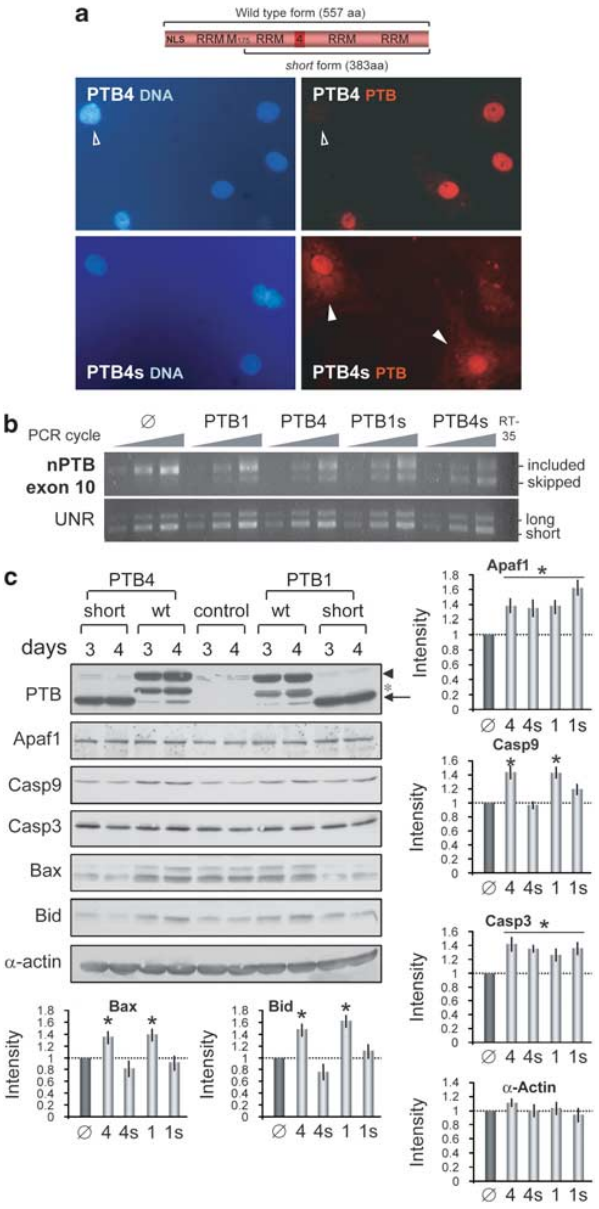

Figure 5 PTB lacking the NLS and RRM1 are partially expressed in the nucleus and induce nPTB exon 10 skipping, yet their capacity for inducing apoptotic gene expression is perturbed. (**a**) Immunofluorescence of postnatal cardiomyocytes overexpressing a short form of PTB lacking the NLS and RRM1 motifs. Images in the figure depict cardiomyocytes overexpressing PTB4 or PTB4 short (identical results were obtained with PTB1/PTB1s, data not shown). DNA was stained with Hoechst dye and PTB expression was immunodetected with an anti-PTB antibody against the c-terminus peptide. Empty arrowhead shows a nontransduced cell where PTB expression is undetectable. White arrowheads show cytosolic PTB expression. (**b**) Semi-quantitative detection of endogenous steady-state nPTB transcript with primers amplifying the exon10 region in reverse-transcribed total RNA extracts from postnatal cardiomyocytes transduced with PTB1, PTB4, PTB1 short, PTB4 short, or empty ($\varnothing$) vectors. A measure of 10 μl samples from different PCR cycles were electroforesed (see Materials and Methods). UNR is used as loading control. RT- sample: the reverse transcription reaction was omitted (cycle 35 is shown). Similar results were obtained in three independent experiments. (**c**) Analysis of the expression of PTB and apoptotic proteins in P4-5 postnatal rat cardiomyocytes transduced with empty lentiviruses (control) or viral particles inducing expression of human wild-type (wt) PTB1, PTB4 or their respective short forms, at days 3 and 4 after transduction. Arrowhead: full-length PTB; arrow: short PTB; *: the intermediate band is probably a product of PTB processing. Densitometric analysis of the western blot signals from three to four independent experiments is expressed as fold *versus* control ($\varnothing$). 1, PTB1; 1s, PTB1 short; 4, PTB4; 4s, PTB4 short. Values are mean corresponding to day four $\pm$S.E.M.; *$P \leqslant 0.05$ *versus* control

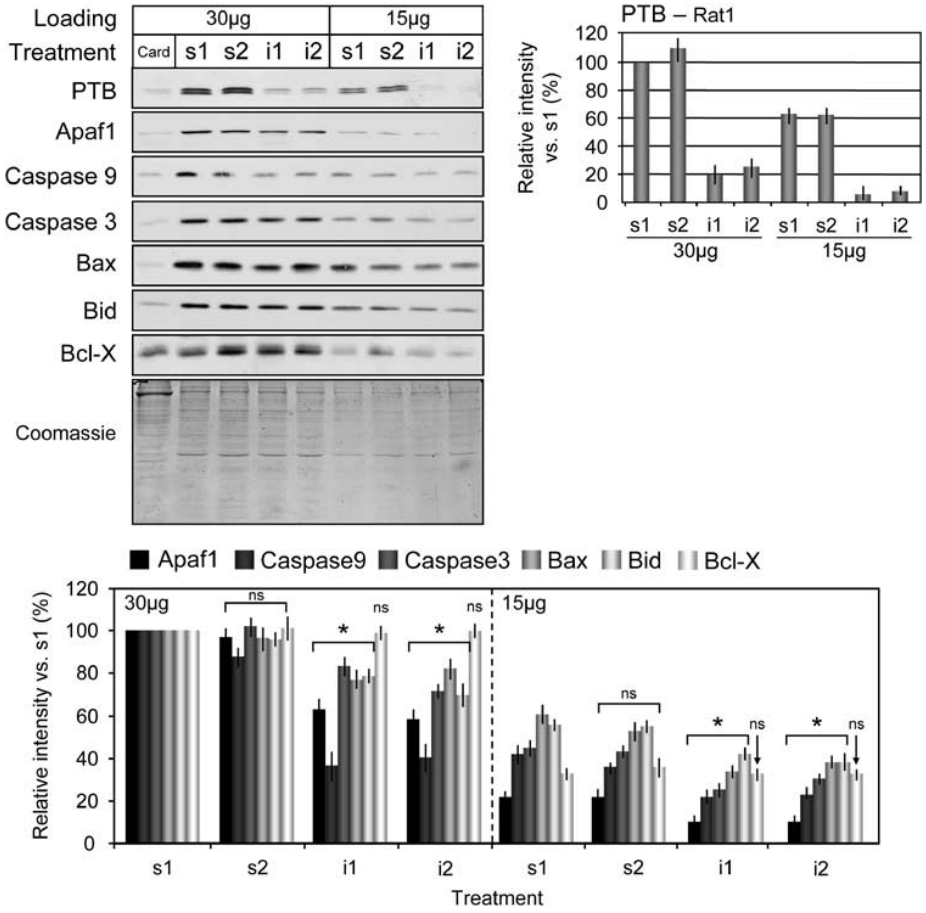

Figure 6 PTB knockdown in Rat-1 fibroblasts induces a general reduction of apoptotic gene expression. Analysis of the expression of PTB and apoptotic proteins in control P4 rat neonatal cardiomyocytes and Rat1 fibroblasts at day four posttransduction with two different shRNA constructs for PTB silencing (i1, i2) or scrambled vectors (s1, s2). In all, 30 and 15 μg of protein lysates were loaded for estimation of the response of the antibodies. Small graph: Densitometric analysis for PTB protein signals expressed normalized to s1 from three independent experiments ($\pm$ S.E.M.). Lower graph: Mean densitometric values of apoptotic protein signals in three to four independent experiments, calculated as described in the Materials and Methods section (Loading: left side, 30 μg protein; right side, 15 μg protein). Statistical significance *versus* S1 is shown. $*P \leqslant 0.05$; ns: not significant

Luciferase (LucF) ORF are separated by the 5′-UTR of Apaf-1. In transfected cells, LucR activity was a measure of transcription and cap-dependent translation and LucF activity required IRES-dependent translation, mediated by PTB in that case. The results presented here using similar bicistronic Luciferase reporter gene constructs suggest that PTB can mediate IRES-dependent translation of Apaf-1 and caspase-3 mRNAs in cardiomyocytes.

We also aimed at analyzing apoptotic gene expression in cardiomyocytes with PTB expression restricted to the cytosol in an attempt to exclude their nuclear functions (i.e. mRNA splicing). We transduced postnatal cardiomyocytes, which express low levels of endogenous PTB, with several PTB constructs harboring mutations or small deletions of amino acids of the N-terminus, reported to be relevant for nuclear targeting in cell lines.[20,21] However, neither single mutation nor mutation of all key residues altered significantly PTB targeting to the nucleus. This suggested that other regions in PTB are important for nuclear expression in primary cardiomyocytes. We decided to delete both the NLS and first RRM because RRM1 has been shown to be important for nuclear localization[21,22] and for protein–protein interactions[28] yet dispensable for PTB–RNA interactions.[21] This short PTB construct conserved the primary determinant for RNA binding specificity, which resides in RRM3 and RRM4, as shown in Vero and HeLa cell lines,[21,29] and the primary contributor to dimer stabilization, which resides in RRM2.[21,29] Unexpectedly, transduction of PTB lacking the NLS and RRM1 in cardiomyocytes induced PTB expression in the cytosol at some extent, yet most of the exogenous PTB was still directed to the nucleus and induced nPTB exon 10 skipping. Nuclear localization of PTB lacking the NLS and RRM1 in primary cardiomyocytes was in apparent contradiction with earlier reports. Indeed, PTB-GST constructs lacking the NLS and RRM1 had been reported to be expressed exclusively in the cytosol of Vero monkey cells,[21] whereas fussing an N-terminus PTB fragment to CAT was sufficient to direct its expression to the cytosol in 3T3 cells, and deletion of GTK or

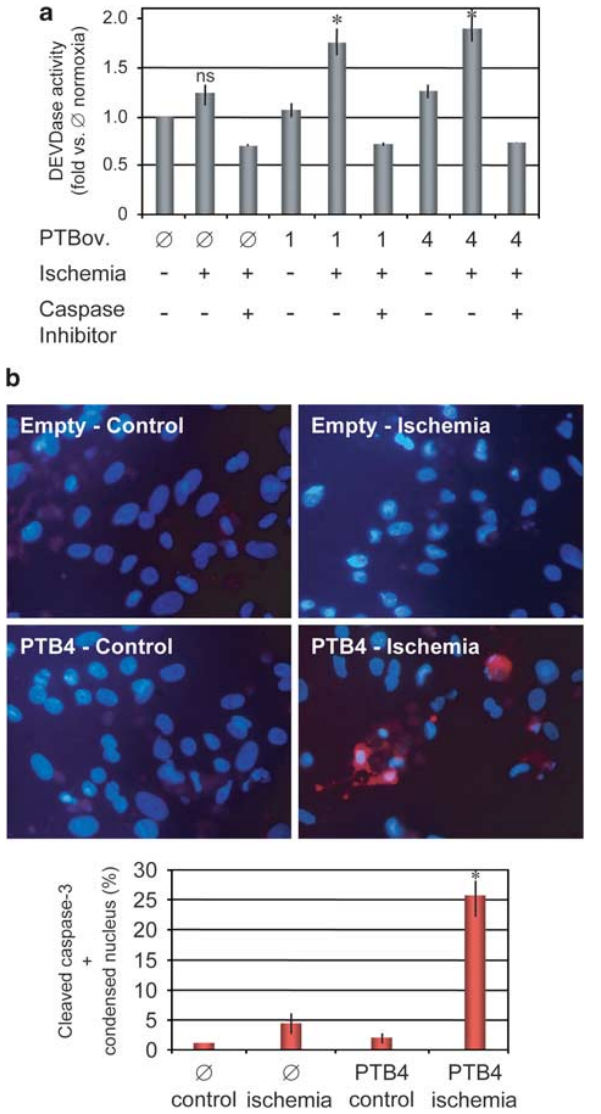

Figure 7 Gene expression changes induced by PTB overexpression in postnatal cardiomyocytes induce a switch from caspase-independent to caspase-dependent apoptosis during experimental ischemia. (**a**) Enzymatic DEVDase activity (as a measurement of executioner caspase activity) was analyzed in cardiomyocytes transduced with empty (∅), PTB1[1] or PTB4[4] vectors and treated with ischemia (see Materials and Methods) in the presence or absence of the pan-caspase inhibitor z-VAD-fmk (100 μM). Values from three experiments performed in duplicates are expressed as fold increase *versus* intra-experiment normoxic cardiomyocytes transduced with empty viruses (± S.E.M.). (**b**) Immunostaining of activated caspase-3 in wild-type and PTB4-overexpressing cardiomyocytes during ischemia. Representative images produced by overlay of the Hoechst staining (chromatin, blue) and anti-cleaved caspase-3 staining (active protease, red) are shown from three independent experiments performed in duplicates. Caspase-dependent apoptosis was quantified by counting cells stained with the activated caspase-3-specific antibody with condensed and or fragmented nucleus and is expressed in percentages as mean ± S.E.M. from three independent experiments performed in duplicates. Statistical significance of changes *versus* values of control cardiomyocytes during ischemia was calculated for (**a**) and (**b**) (*$P \leqslant 0.05$ *versus* empty virus)

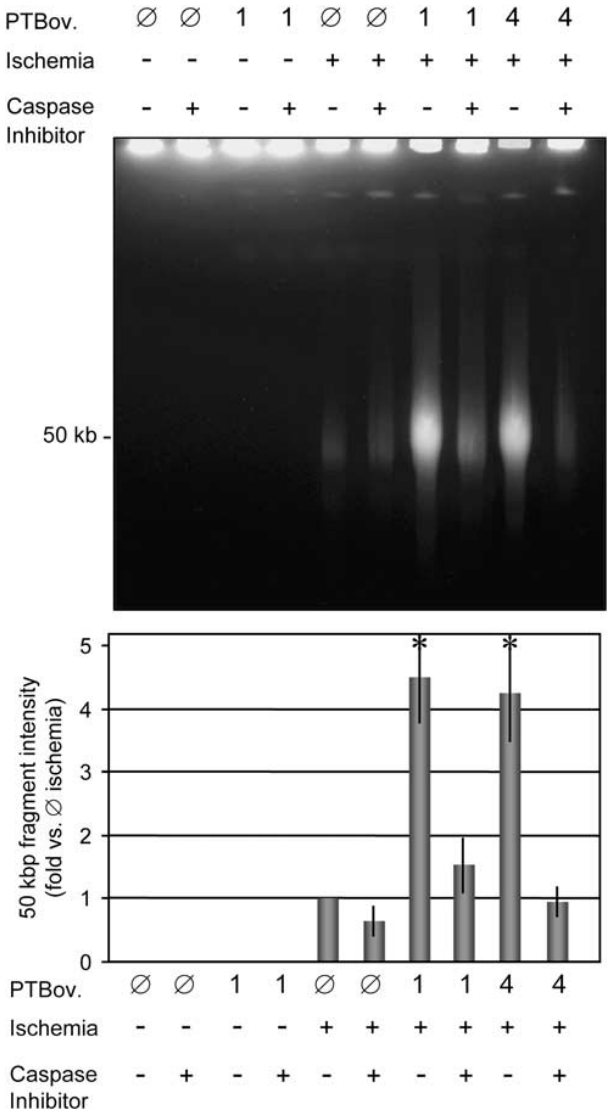

Figure 8 PTB overexpression in postnatal cardiomyocytes induce the activation of caspase-dependent DNA damage during experimental ischemia. High molecular weight DNA fragmentation was analyzed by pulse-field electrophoresis in DNA samples from cardiomyocytes transduced with empty (∅), PTB1[1] or PTB4[4] vectors and cultured in standard conditions or treated with experimental ischemia (see Materials and Methods). Densitometric analysis of the region around the 50 kb size was performed and values were expressed as fold *versus* cardiomyocytes transduced with empty viruses and treated with ischemia. Bars represent mean of three independent experiments ± S.E.M.; *$P \leqslant 0.05$ *versus* cardiomyocytes transduced with empty viruses in the same experimental conditions

KKF motifs of the N-terminal region restricted CAT expression to the cytosol.[20] One difference between this study and other's reports, in addition to the use of primary cells instead of cell lines, is that we have analyzed PTB distribution with anti-PTB antibodies and not by detection of a long tag fussed to the protein or a PTB fragment. Our approach implies the possibility of detecting short PTB-induced expression of endogenous full-length PTB that would be directed to the nucleus. However, this was discarded by western blot analysis showing

no changes in endogenous PTB expression in these experiments. Finally, abnormal behavior of PTB distribution due to overexpression in cardiomyocytes that does not happen in overexpressing cell lines is improbable because, in our hands, overexpression of other genes using identical procedure did not alter their expected subcellular distribution (ref. [7] and data not shown). Thus, these results suggest that other regions outside the NLS and RRM1 of PTB are relevant for its intracellular localization in cardiomyocytes and/or that PTB can associate with other proteins to enter the nucleus without requiring the NLS. These results also showed that NLS and RRM1 of PTB are important for inducing expression of a subset of apoptotic genes (e.g. Caspase-9, Bax, and Bid), but not for the regulation of other target genes (e.g. Apaf-1 and Caspase-3).

Our results also show that the slight increase in apoptotic gene expression induced by PTB, probably in addition to modifications in the expression of other target genes relevant for apoptosis, is sufficient for changing the main pathway leading to cardiomyocyte damage during stress (i.e. from caspase-independent DNA damage to caspase activation and caspase-dependent DNA damage). These results show that small changes in apoptotic gene expression modify the susceptibility of cardiomyocytes to apoptotic stimuli, and point to PTB as relevant regulators in the control of the susceptibility of differentiating cardiomyocytes to caspase-dependent apoptosis through posttranscriptional regulation of apoptotic gene expression, involving IRES-dependent translation.

Materials and Methods

Animals, tissues, and cell cultures. The investigation with experimental animals conforms to the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication No. 85–23, revised 1996) and was approved by our Experimental Animal Ethic Committee. Sprague–Dawley rat's hearts were dissected minced into small cubes, rinsed with cold phosphate-buffered saline and snap-frozen into liquid nitrogen. Rat neonatal cardiomyocytes were obtained from the heart of 4–5-day-old pups and adult cardiomyocytes were isolated from 3-month-old male rats as described elsewhere.[7] Rat-1 rat fibroblast cell line was cultured in DMEM medium supplemented with 10% Fetal Calf Serum (Invitrogen, SA, Spain).

Lentiviral construction and infection. Human PTB1 and PTB4 cDNAs (from Chris W Smith, Cambridge, UK) were cloned into pEIGW vector (from Didier Trono, Geneva, Switzerland) using the SmaI and NdeI sites. Using pEIGW-PTB1/4 as templates, short forms of PTB1/4Δ1-174 were amplified, sequenced, and cloned into the pEIGW vector in-frame with SmaI and NdeI sites. Specific 19 nucleotide sequences were chosen for PTB using the free RNAi design interfaces (see Supplementary Table 1 for details). Primers for small hairpin RNA interference (shRNA) were subcloned into the pLVTHM plasmid as described earlier.[7] pEIGW or pLVTHM constructs were transfected into HEK293T cells together with the plasmids psPAX2 and pMD2G (from Didier Trono) to produce viruses. Cells were usually treated after 3 to 6 days of transduction as described elsewhere in detail.[7]

Hypoxic treatment. Experimental ischemia was achieved by culturing cells in Tyrode's solution (NaCl 137 mM, KCl 2.7 mM, Na2HPO4 8 mM, KH2PO4 1.5 mM, CaCl2 0.9 mM, and 0.5 mM, initial pH: 7.2; all reagents from SIGMA-ALDRICH, Spain) inside a Ruskin In Vivo2 400 Hypoxia Workstation (Ruskin, UK) in a mixture of 5% CO_2 and 95% N_2 following manufacturer's instructions to attain a 0.2% oxygen concentration. Pan-caspase inhibitor Z-Val-Ala-Asp(OMe)-CH2F (Z-VAD-fmk) (Merck-Calbiochem) was used at a concentration of 100 μM.[7]

Western blot, RT-PCR, and immunofluorescence. Protein expression was detected in protein extracts diluted in Tris-buffered 2% SDS solution at pH 6.8 as reported earlier.[30] Antibody data can be found in the Supplementary Table 2. Each immunodetection was repeated with three to four different membranes containing samples coming from different animals of each age or from cell cultures, as described in each figure legend. Immunoblots were developed with the ECL chemiluminescence detection kit (Biological Industries, Israel). Different exposures were performed on SuperRX Fuji Film (Fuji, Tokyo, Japan) to obtain nonsaturated signals. Densitometry was performed with the Scion Image software (Scion Corporation, Frederick, MD, USA) and qualitative calculations were done comparing data from nonsaturated film images. Special care was taken to assure that antibody dilutions and conditions of immunodetection allowed getting signals in the linear range of antibody response (see Supplementary Figure 2). Total RNA was obtained from frozen tissues or cell pellets with the RNeasy Mini Kit (Qiagen, Spain). RNA concentration measurements and reverse transcription were done as described.[7,8] Equal volumes of cDNA were amplified by PCR in 50 μl of reaction mix using a couple of specific primers expanding at least two exons within the gene of interest (see Supplementary Table 1 for details) as described elsewhere.[7,8] Aliquots (10 μl) were taken at PCR cycles 25, 30, and 35, mixed with SYBR Safe dye (Invitrogen) and migrated in a 3% agarose gel. Densitometry of the DNA bands was performed with the Scion Image software (Scion Corporation, Washington, USA) and qualitative calculations were done comparing data from nonsaturated products. Loading was checked by amplification of the UNR transcript. Transcript analyses were performed from three samples coming from different animals and cell cultures. PTB expression was also analyzed by immunofluorescence in two independent cardiomyocyte cultures after fixing in 4% paraformaldehyde as described earlier[7] using the antibody against the PTB c-terminal region (see Supplementary Table 2) at 1 : 5000 dilution. Caspase-3 processing required for its activation was analyzed with a cleaved caspase-3-specific antibody (Cell Signaling, Danvers, MA, USA) following the above procedure. Hoechst staining at the end of the IF procedures was performed to observe nuclear morphology. Each treatment was performed in duplicates in two independent experiments.

Enzymatic caspase activity assay. Executioner caspase enzymatic activity was measured in cell extracts as reported earlier[30] using the fluorogenic substrate Z-DEVD-AFC (Caspase-3 Substrate IV; Merck-Calbiochem) at 50 μM in 96-well plates. Fluorescence was detected with a Biotek Instruments Inc. FL $\times$ 800 Fluorescence Reader (Izasa, Spain) with excitation filter set at 360 nm and emission filter at 530 nm. Data obtained for different experimental conditions were compared within the linear phase of absorbance increase. Data are mean of three independent experiments performed in duplicates.

Luciferase reporter gene assay using bicistronic plasmids. The 5′-UTR region of Apaf-1 and Caspase-3 human genes were PCR-amplified from reverse-transcribed 293T human cell line total RNA, using primers designed from the gene sequences available at GenBank with accession numbers NM_181861 (Apaf-1) and NM_004346 (Caspase-3). Each 5′-UTR was subcloned into the pCRF1AL+ bicistronic plasmid[31] between the SpeI and NcoI sites (NcoI is the AUG codon of LucF+). The 5′-UTRs were sequenced to confirm perfect matching with the published sequences. Transcription of the bicistronic transcript is regulated by the CMV promoter and the LucR gene, which lies upstream of the LucF+ gene, is translated by a cap-dependent mechanism and is used as control of transfection/transcription. LucF+ translation occurs only if preceded by a sequence mediating IRES-dependent translation.[12,15,31] pCRHL vector, harboring a short hairpin sequence between LucR and LucF+ was used as positive control for LucR and negative control for LucF, whereas pSV40LucF, where LucF is under the direct control of SV40 promoter was used as a positive control for LucF and negative for LucR. Four hours after seeding, 10 μg of bicistronic plasmid was transfected into neonatal cardiomyocyte cultures (0.95×10^6 cells/P35-plate) with Lipofectamine 2000 (Invitrogen) following manufacturer's instructions. A few minutes later, cells were transduced with control lentiviruses or lentiviruses inducing expression of PTB4. Four days later, two cultures per treatment were harvested, washed in cold PBS, lysed in passive lysis buffer and frozen. Quantification of luciferase activity was performed with the Dual-Luciferase Reporter Assay System (Promega, Madison, WI, USA) following manufacturer's instructions and luminescence was detected with a Biotek Instruments Inc. FL $\times$ 800 Fluorescence Reader (Izasa, Spain) set at 620 nm emission wavelength. Values were normalized to the luciferase activity detected in cells transfected with pCRHL. Statistical significance of differences of normalized values coming from four experiments was calculated by the Student's *t* test.

DNA integrity assay. Cells were pelleted at the end of each treatment and frozen at $-80\,^{\circ}$C. Pellets from the same experiment were processed at once.

They were diluted in 40 μl of sterile phosphate-buffered saline, mixed with 40 μl of melted 1% low melting agarose (Sigma) in 0.5 $\times$ TBE (45 mM Tris pH 8.3, 45 mM boric acid, 1.0 mM EDTA; all reagents from SIGMA). Each mixture was poured into a block caster and let solidify. Each agarose block was submerged into 1 ml of lysis buffer (1% lauryl sarcosil, 0.5 M EDTA, 10 mM Tris, pH 8, 100 μg/ml proteinase K; all reagents from SIGMA) at 50 °C during 24 h in mild agitation, and rinsed twice with 0.5 $\times$ TBE for 1 h at room temperature. For analysis of DNA high molecular weight degradation, the blocks were then laid into wells of a 1% agarose, 0.5 $\times$ TBE gel (CHEF grade, SIGMA-Aldrich). Pulse field electrophoresis was performed as described earlier[7] in a CHEF DR-II system (Bio-Rad, Hercules, CA, USA) set to the following protocol: run time, 14 h; switch time from 5 to 50 s; voltage gradient, 6 V/cm. Gels were stained with SYBR Safe (Molecular Probes, Carlsbad, CA, USA, Invitrogen), visualized by UV exposure and recorded with a Kodak DC290 digital camera. Densitometry was performed as described above.

Statistical analysis. Data are expressed as mean $\pm$ s.e.m. of three or four independent experiments or as specified for each figure. The significance of differences among means was evaluated using the Student's *t* test. A value of $P \leqslant 0.05$ was considered statistically significant.

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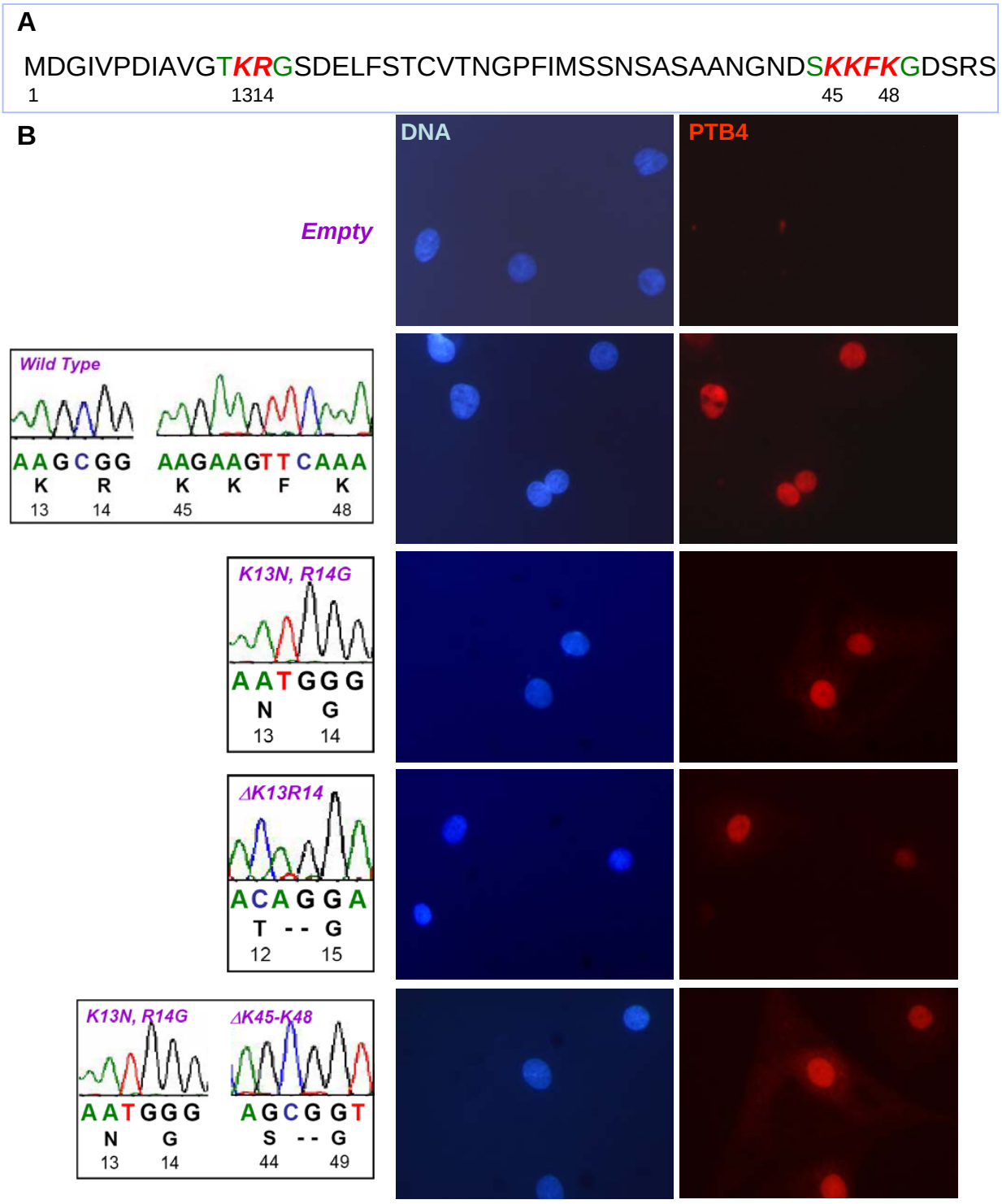
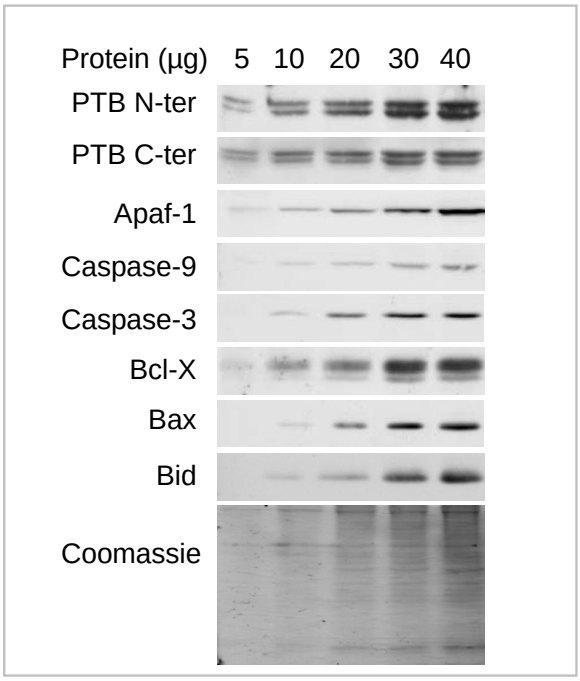
Supplementary Information accompanies the paper on Cell Death and Differentiation website (http://www.nature.com/cdd)

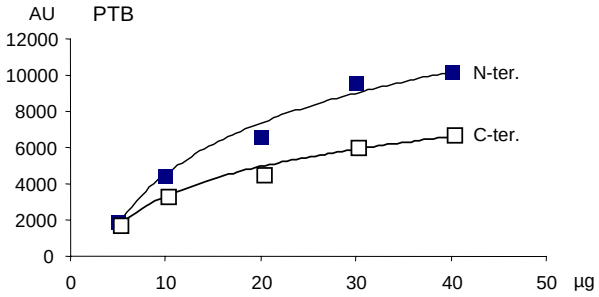
Supplemental Figure S1. **Mutations of amino acids reported to be essential for PTB nuclear localization in cell lines do not modify significantly PTB targeting to the nucleus in primary rat neonatal cardiomyocytes.**

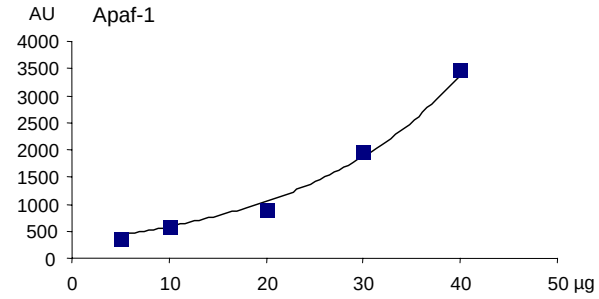


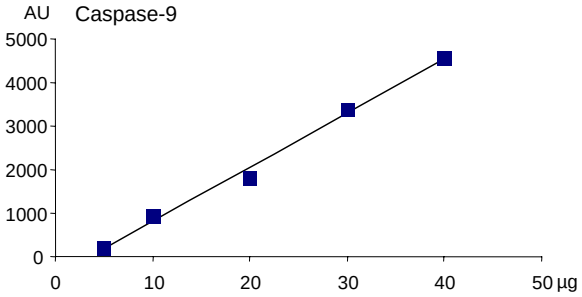
A) Sequence of human PTB protein N-terminal region identical in PTB1 and PTB4 (from NCBI Accession number NP_002810). Residues K13, R14 and KKFK (45-48) have been described to be important for PTB targeting to the nucleus in cell lines (Romanelli et al 1997). B) Postnatal cardiomyocytes were transduced with lentiviruses carrying empty vector (Empty), PTB4 wild type (Wild Type), PTB4 harboring mutations K13N and R14G (K13N R14G), harboring a deletion of K13 and R14 (ΔK13R14), or harboring mutations K13N and R14G and deletion of K45 to K48 (K13N, R14G ΔK45-K48). Sequencing electropherograms depicting the changes introduced in PTB are shown on the left. Representative images of cells transduced with each vector are shown in the panels on the right. Four days after transduction, cells were fixed with PFA 4% and immunostained for PTB as described in the Experimental Procedures section. Blue: DNA staining with Hoechst; Red: PTB immunostaining with an antibody against the PTB c-terminal region.These experiments were carried out also with PTB1 wild type and mutated constructs with same results.

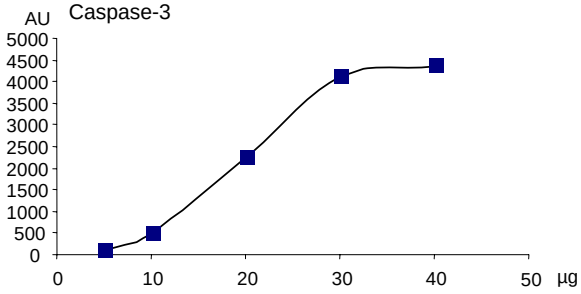
Supplemental Figure S2. Antibody titering

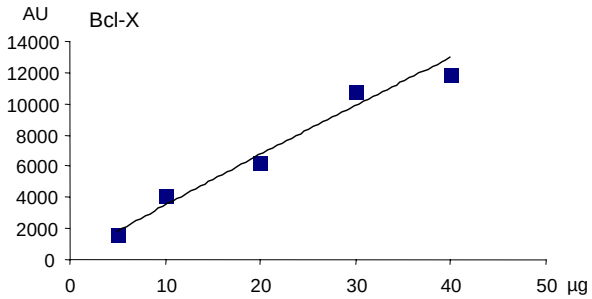


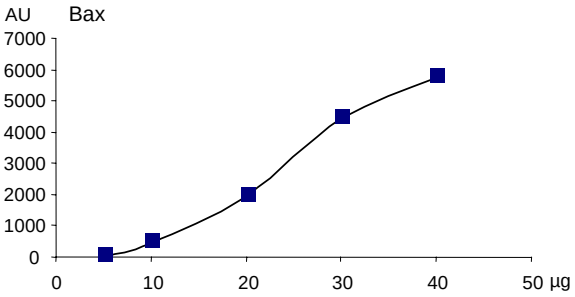


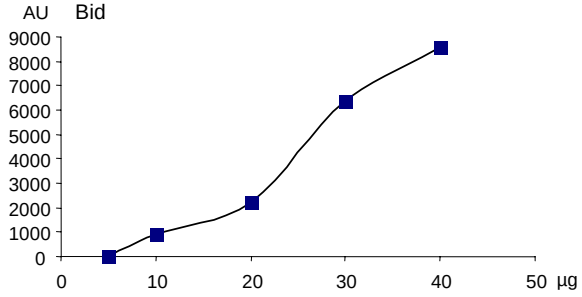












Different volumes of total SDS extracts from 293 cells, ranging from 5 to 40 µg of protein, were separated by SDS-PAGE, transferred to PVDF membranes and incubated with different antibodies as described in the M&M section. Densitometry of the bands was performed as described in the M&M section and densitometric values were plotted against protein concentration.

AU: arbitrary units.

Supplemental Table S1. **Primers for subcloning, RT-PCR and shRNA**

Target gene	Forward (5'-3')	Reverse (5'-3')
PTB1/4 Rn	ACAACGACAAGAGCCGAGAC	GGCTCCATGGACATTAGGAA
PTB1/4 Hs	GTGACTACACACGCCCAGAC	TGACCAGCAATACAGAATTTCC
Apaf-1 Rn	TGCAGATGGCGACAGAATAATAG	CCGTGGACCCAACTTAAATG
Caspase-3 Rn	AACCTCAGAGAGACATTCATGG	GAGTTTCGGCTTTCCAGTCA
Caspase-9 Rn	CTCCTGCGGCGATGC	CCACTGGGGTGAGGTTTC
Bax Rn	TGCAGAGGATGATTGCTGAC	GATCAGCTCGGGCACTTTAG
Bid Rn	AGCAGGTGATGAACTGGACC	AGACGTCACGGAGCAGAGAT
Bcl-XL Rn	CATATAACCCCAGGGACAGC	GTCATGCCCGTCAGGAAC
nPTB Rn exon10	TGTCCCAGGAGCTCTCAGTC	AGCTGTGACTGGTTCCCATC
nPTB Rn	GGTTGCTGTTGGTGTGAAGAG	GCTTCCTCTGTTGCCAGTTC
α-actin	CACGGCATTATCACCAACTG	AACAATGCCTGTGGTTCTCC
UNR Rn	ATCATCTGACCGGAGGACTG	TGAACGTTCCCTTCCCATC
pEIGW-s PTB1/4	GTCCAGTCGGGGAACCTG	ACCGGCCTTATTCCAAGC
PTB* (RNAi 1)	GCACAGTCCTGAAGATCAT	--
PTB* (RNAi 2)	GCTGCCAACACTATGGTTA	--
PTB (Scr 1)	GCCCCTCCGATAAAGATTG	--
PTB (Scr 2)	GCGTCGTCAAAGGATTCAA	--

Rn: Rattus norvegicus
Hs: Homo sapiens
* Only the target sequence is shown.
Scr: scrambled sequence.

Supplemental Table S2. **Antibodies**

Antigen	Provider	Catalogue Number	Dilution	Incubation
PTB(N-terminus)	Abcam	ab5642	1:5000	O.N. 4°C
PTB(C-terminus)	ZYMED	32-4800	1:15000	1h RT
Caspase-3	Cell Signaling	9665	1:5000	O.N. 4°C
Caspase-9	Cell Signaling	9508	1:5000	O.N. 4°C
Apaf-1	Alexis	alx-804-349	1:5000	O.N. 4°C
Bax	Cell Signaling	2772	1:5000	O.N. 4°C
Bid	R&D System	AF860	1:5000	1h RT
Bcl-X	BD Transduction	610212	1:5000	O.N. 4°C
α-Actin	Sigma	A2172	1:160000	20 min RT

O.N. overnight
RT Room Temperature

All secondary antibodies were used at 1:10000 1 hour at room temperature, except for the secondary antibody against α-actin antibody that was incubated 20 min.

Discussion

Silencing of apoptotic gene expression during heart development

We have found that the genes controlling Caspase-dependent cell death are expressed in the embryonic heart, brain and liver, but are later silenced during postnatal development, especially in the heart and the brain. Of note, the expression of many genes controlling intermediate steps leading to Caspase activation is always low in the liver, whose sensitivity to Caspase-dependent cell death could depend on the high expression of Bid and executioner Caspase-3 and -7. The gene silencing event implies a reduction in the amount of the transcripts coding for these proteins.

Although previous reports have shown that the expression of some apoptotic proteins is reduced in postnatal animals (Yakovlev, Ota et al. 2001; Donovan and Cotter 2002; Wright, Linhoff et al. 2004; Bahi, Zhang et al. 2006; Donovan, Doonan et al. 2006; Stoka, Turk et al. 2006), simultaneous data argue in favour of high expression of some of these proteins (Yakovlev, Ota et al. 2001; Wright, Linhoff et al. 2004; Potts, Vaughn et al. 2005; Han, Chen et al. 2006; Stoka, Turk et al. 2006; Madden, Donovan et al. 2007). From our point of view, clarifying this fact has important consequences for the correct understanding of the mechanisms engaged in cardiomyocyte and neuron cell death in the adult. Indeed, outstanding research on neurodegenerative and cardiac diseases is being frequently conducted or complemented with studies performed with embryonic cells and immortalized cell lines, which express the full Caspase-dependent death machinery. Using these models, Caspase activation has been proposed to play a relevant role in cardiomyocyte and neuron death during disease (Gervais, Xu et al. 1999; Hermel, Gafni et al. 2004; Nam, Mani et al. 2004). However, although the role of Caspase-dependent events on the morphogenesis of the brain and the heart are well established (Cecconi, Alvarez-Bolado et al. 1998; Yoshida, Kong et al. 1998; Lakhani, Masud et al. 2006), the role of Caspases in postmitotic cell death is controversial (Ohno, Takemura et al. 1998; Knaapen, Davies et al. 2001; Chen, Won et al. 2002; Donovan and Cotter 2002).

Our results show that the pro-death genes in the Caspase-dependent pathway are repressed at both protein and transcript levels during development, in apparent contradiction with previous reports (Potts, Vaughn et al. 2005; Han, Chen et al. 2006). Although reduction in Apaf-1 expression and relatively unchanged levels of Caspase-9 are observed when comparing embryos with early neonatal

cardiomyocytes (Potts, Vaughn et al. 2005), in the present work we show that adult fully differentiated cardiomyocytes lack both proteins. A previous work showed that Caspase-9 expression increases during heart development (Han, Chen et al. 2006). However, the size of the Caspase-9 band shown in that report was smaller than expected and varied in size when comparing heart and brain extracts. We checked the specificity of two commercial antibodies against Caspase-9. We found that only one antibody detected correctly the different size of the human and rodent Caspase-9 isoforms. This antibody detected Caspase-9 only in embryonic and at lesser extent in young heart, but not in adult heart and adult isolated cardiomyocytes. Therefore, we conclude that Caspase-9 is actually silenced during development in heart and brain, alike the rest of Caspases analyzed. Caspase-9 has been suggested to be expressed in adult mouse heart both at the protein and transcript levels (Madden et al., 2007). In support of the decrease in Caspase-9 expression in the myocardium, we report here that isolated adult cardiomyocytes are virtually devoid of Caspase-9, suggesting that the remnant Caspase-9 signal in adult heart comes from non-muscular cells. In addition, we have taken into account only nonsaturated signals for our comparative transcript analysis, which confirm an important decrease in Caspase-9 transcript both in the heart and the brain.

Our results also show that expression of apoptosis inducing factor (AIF), a potentially deleterious protein, is increased in the adult heart. Interestingly, we and others have demonstrated that AIF plays actually a beneficial role during reoxygenation in cardiomyocytes (van Empel, Bertrand et al. 2005; Bahi, Zhang et al. 2006), and protects the heart and the brain against oxidative damage (Klein, Longo-Guess et al. 2002; van Empel, Bertrand et al. 2005). Another interesting fact is that the expression of pro-death proteins Bax and Bak, thought to play redundant roles, vary when comparing different tissues. While both Bax and Bak are expressed in the young heart, Bak is the major Bcl-2-related pro-death member in the brain, whereas Bax is more expressed than Bak in the liver. These results suggest that the relevance of Bak and Bax in the control of the mitochondrial events leading to cytochrome c release is tissue-specific. Finally, our results show that Bcl-XL expression is sustained at significant levels until adulthood, suggesting that it could be a crucial protector of mitochondrial integrity in the adult brain and heart.

Switch from Caspase-dependent cell death to Caspase-independent cell death during heart development

During an ischemic insult, postnatal cardiomyocytes do not activate Caspases as readily as other cell types (Scarabelli, Stephanou et al. 2002; Kostin, Pool et al. 2003). Our results also show that the ischemia induced activation of Caspase-3 can not be detected in neonatal cardiomyocytes compared with the embryonic cardiomyocytes. The developmental repression of Caspase-3 activation strongly suggests the switch from Caspase-dependent cell death to Caspase-independent cell death during heart development. However, the underlying molecular mechanisms are not well understood because cardiac cells have been assumed to express a functional Caspase-dependent pathway. Several molecules have been described to protect cardiomyocyte mitochondria by blocking cytochrome c release, and have been proposed to be apoptotic blockers in cardiomyocytes. Heat shock proteins have been suggested to be endogenous protectors against ischemic damage in cardiomyocytes (Marber, Mestril et al. 1995; Nakano, Mann et al. 1997) by protecting mitochondrial function (Gupta and Knowlton 2002). Experimental overexpression of Bcl-2 or Bcl-XL, which block cytochrome c translocation, have been reported to be cardioprotective during ischemia/reoxygenation (Chen, Chua et al. 2001; Chatterjee, Stewart et al. 2002; Huang, Ito et al. 2003). Accordingly, the hearts of mice deficient for Bax, which induces cytochrome c release, are partially protected during ischemia (Hochhauser, Kivity et al. 2003). Apoptosis Repressor with Caspase Recruitment Domain (ARC) is highly expressed in the heart (Ekhterae, Lin et al. 1999), and protects this organ against ischemic-induced cell death (Gustafsson, Sayen et al. 2002). ARC blocks cytochrome c release by interfering with Bax (Gustafsson, Tsai et al. 2004; Nam, Mani et al. 2004). From the above-mentioned observations, it has been assumed that activation of the mitochondrial apoptotic pathway leading to executioner Caspase activation is relevant in the cardiac ischemic damage. However, Caspase activity was not measured (Gustafsson, Sayen et al. 2002), or it was only analyzed in the heart-derived cell line H9c2 (Ekhterae, Lin et al. 1999; Gustafsson, Tsai et al. 2004; Nam, Mani et al. 2004). Importantly, heart-derived cell lines H9c2 and HL-1 (Claycomb, Lanson et al. 1998) are not terminally differentiated cells and, contrary to cardiomyocytes, express all the apoptotic regulators.

Furthermore, it is unknown why in conditions where Caspase-dependent programmed cell death should be engaged (*i.e.* after cytochrome *c* release (Akao, O'Rourke et al. 2003)); there was only low Caspase activation in cardiomyocytes (Teshima, Akao et al. 2003). What blocks cardiomyocyte apoptosis after cytochrome *c* release? We have previously described that cardiomyocytes lack significant Apaf-1 expression (Sanchis et al., 2003), and this could hamper the activation of Caspase-9 after cytochrome *c* release. A recent report proposes that the low expression of Apaf-1 helps inhibitor of apoptosis proteins (IAPs) to better counteract executioner Caspase activation in cardiomyocytes, which were suggested to express Caspases at a similar level than fibroblasts (Potts, Vaughn et al. 2005). Contrary to this work, the data reported here demonstrate a global down-regulation of the whole Caspase-dependent machinery in the heart during development. Reduction in Caspase expression is evident when comparing pure isolated embryonic and adult cardiomyocytes, suggesting that most of the Caspase expression in neonatal cardiomyocyte cultures comes from dividing cardiac fibroblasts, which usually contaminate cardiomyocyte cultures. In addition, our results discard an inhibition of Caspase activation by ischemia-induced acidosis, because fibroblasts and embryonic cardiomyocytes do activate Caspases in this setting, even at pH 6.1. Therefore, we propose that differentiation-related repression of the whole Caspase-dependent machinery accounts for postnatal cardiac protection against Caspase activation. We hypothesize that down-regulation of the apoptotic machinery during differentiation can be a mechanism for reducing Caspase-induced cell damage by accidental cytochrome *c* translocation due to the high number of mitochondria and the constant change of cardiomyocyte volume during the contraction/ relaxation cycle.

EndoG contributes to the ischemia induced DNA fragmentation in cardiomyocytes

Given the importance of switch from Caspase-dependent cell death to Caspase-independent cell death in neonatal cardiomyocytes, we try to clarify the mechanism unrelated to Caspase activation. We investigated the ischemia induced DNA fragmentation, one of the hallmarks of apoptosis, in neonatal cardiomyocytes. Our results demonstrate that cytochrome *c* translocation during cardiac ischemia is not

linked to the Caspase-dependent death pathway, contrary to the dogma that has been established in recent years. Nevertheless, mitochondrial damage is involved in ischemia-induced cardiomyocyte cell death and hampers cardiac recovery during reoxygenation. In this respect, truncated-AIF translocation from mitochondria was detected in ischemic cardiomyocytes. Of note, pharmacological calpain inhibition did not prevent ischemia-induced AIF cleavage and release. This fact discards an essential role of calpains in AIF translocation and release in ischemic cardiomyocytes. In our experimental model, AIF release did not account for ischemia-induced cardiac DNA damage. Furthermore, we show that AIF depletion hampers cardiac survival during reoxygenation. Importantly, the ischemic lesion is bigger in the AIF-defective Harlequin mouse (van Empel, Bertrand et al. 2005). In addition, we have recently shown that AIF is not related to DNA damage in staurosporine-treated neuroblastoma cells (Yuste, Sanchez-Lopez et al. 2005). Therefore, our data suggest that AIF role in DNA cleavage depends on the cell type and does not support a relevant role for AIF in cardiac DNA processing.

The present work also reveals that EndoG is the main contributor for the DNA low molecular weight degradation in ischemic cardiomyocytes. Interestingly, two recent works reported no obvious effects on cell death and DNA degradation in EndoG-deficient mice (Irvine, Adachi et al. 2005; David, Sasaki et al. 2006). However, DNA integrity analysis was studied in splenocytes, which otherwise activate Caspase-dependent apoptosis (Irvine, Adachi et al. 2005). It must be taken into account that Li *et al.* showed the EndoG role in mammalian apoptotic DNA degradation in fibroblasts deficient for Caspase activated DNase (Li et al., 2001). Furthermore, here we show that EndoG silencing in primary skin fibroblasts, which express Caspases, does not induce a reduction in DNA cleavage. Taken together, these results suggest that EndoG cleaves DNA in systems where Caspases are absent, irrespective of the death stimulus, and could be the final step of DNA processing in postmitotic cells. Finally, our results show that high molecular weight fragmentation of DNA is Caspase-independent in dividing fibroblasts and postmitotic cardiomyocytes and that AIF is involved in this step of DNA degradation only in proliferating skin fibroblasts but not in cardiomyocytes. Indeed, a functional genomic approach in *C. elegans* has highlighted the involvement of many genes in

the final apoptotic DNA degradation process, which could be controlling the final cell fate (Parrish and Xue 2003).

Bcl-XL hampers and BNip3 contributes to EndoG release from mitochondria

A great advance in the knowledge about the events contributing to ischemia-induced heart disease has been achieved in recent years and, in particular, about the role of cardiomyocyte cell damage in heart failure (Foo et al., 2005). However, in our opinion some confusion still remains about the intracellular mechanisms involved in cardiomyocyte damage during ischemia and ischemia/reperfusion. Here, we show that ischemia-induced TUNEL-positive DNA damage in ischemic cardiomyocytes *in vitro* is triggered by EndoG and depends on EndoG release from mitochondria induced by BNip3 in a way that can be blocked by Bcl-XL, without the involvement of Caspases.

The contribution of the canonical Caspase-dependent apoptotic pathway to ischemia-induced cardiomyocyte cell death was initially described by Tanaka et al., 1994. The extrinsic, death receptor-dependent, apoptotic cascade (Lee et al., 2002 and many others) and the mitochondria-dependent pathway of apoptosis (reviewed by (Crow et al., 2004) have been suggested to contribute to cardiomyocyte death during ischemia or ischemia/reperfusion. However, simultaneous reports showed that myocyte damage and death were unrelated to Caspases (Bahi et al., 2006; Chen et al., 2002; Knaapen et al., 2001; Kubasiak et al., 2002). The lack of Caspase activity in ischemic cardiomyocytes is in agreement with our previous finding that expression of the apoptotic genes is silenced during cardiomyocyte differentiation (Bahi et al., 2006; Zhang et al., 2007a). Using different approaches, it has been shown that Bcl-2 and Bcl-XL could blunt ischemia-induced cardiomyocyte damage (Chatterjee et al., 2002; Chen et al., 2001b; Huang et al., 2003), also suggesting the involvement of the Caspase-dependent pathway triggered by cytochrome c translocation. The use of the TUNEL assay and DNA electrophoresis to assess DNA fragmentation as reliable methods for detecting apoptotic myocytes in human samples contributed to the knowledge of the role of cell damage in heart failure but

biased the interpretation of the mechanisms involved in this event (Narula et al., 1996; Olivetti et al., 1997; Saraste et al., 1997; Veinot et al., 1997).

Although many excellent works have unveiled the essential role of BNip3 in DNA damage in myocytes and other cell types (Guo et al., 2001; Kubasiak et al., 2002; Lamy et al., 2003; Vande Velde et al., 2000), the nuclease executing BNip3-dependent DNA damage has not been identified so far. Here we show that endogenous BNip3 induces EndoG release and Caspase-independent EndoG-dependent DNA fragmentation, supporting a role of EndoG as the executioner of DNA damage triggered by BNip3. We also extent the knowledge about the role of BNip3 in DNA damage by showing for the first time that high molecular weight DNA fragmentation also depends on BNip3 activity in ischemic cardiomyocytes. In addition, although cell damage induced by BNip3 depend on Bax and Bak activation and induce Caspase activation in Caspase-dependent cellular models such as embryonic fibroblasts (MEF) and the immortalized embryonic atrial myocytes (HL-1) (see review in (Burton and Gibson, 2009), our results give support to a role of BNip3 unrelated to Caspases in differentiated cardiomyocytes as previously shown by others (Kubasiak et al., 2002) and also suggest a Caspase-independent mechanism for the known protective role of overexpressed Bcl-XL in the heart *in vivo* (Huang et al., 2003).

The link between TUNEL-positive DNA fragmentation and Caspase activity, which is true for other cellular models and that has been assumed for cardiomyocytes, was questioned by investigations (French et al., 2009; Knaapen et al., 2001). Our data demonstrate that the TUNEL signal is produced by the activity of EndoG without the contribution of Caspases in ischemic cardiomyocytes.

Our results provide evidence that EndoG is the link between BNip3-mediated mitochondrial dysfunction and DNA degradation during ischemia and that this pathway is unrelated to Caspase activation in cardiomyocytes. In addition, we show that TUNEL labeling of ischemic cardiomyocytes, which has been used frequently as a marker of Caspase-dependent apoptosis, is due to EndoG and is unrelated to Caspase activity in these cells. Finally, our results show that Bcl-XL can prevent ischemia-induced DNA fragmentation by blocking EndoG translocation from mitochondria. Thus, the evidence that BNip3 and EndoG form a pathway for large-scale and low molecular weight DNA degradation in cardiomyocytes unrelated to

Caspase activation add information to previous reports supporting Caspase-independent mechanisms during cardiac ischemia and ischemia-reperfusion, clarify the induction of Caspase-independent DNA damage by BNip3, demonstrate that the TUNEL assay detects Caspase-independent, EndoG-dependent DNA damage in ischemic myocytes and agree with a type of cell damage unrelated to Caspases that can take place after the global expression silencing of genes of the Caspase-dependent signaling cascade during myocyte differentiation (Bahi et al., 2006; Doss et al., 2007; Zhang et al., 2007a).

E2F factors are not involved in the expression of the major apoptotic genes neither during stress nor after an increase in E2F1 expression.

We have shown the developmental silencing of key apoptotic proteins in the heart. It is interesting to find some potential regulators which control one or most of apoptotic gene expression. We have found that E2F expression decreased during heart development, which is consistent with the downregulation trends of apoptotic proteins. We thought, thus, this transcription factor could be one of the potential candidates. Unfortunately, expression models of apoptotic regulators are similar compared E2F1, E2F2 and E2F1/2 double knockout mice with the wild type. Regarding the mechanisms involved in apoptotic gene downregulation, our data discard an essential role of E2F1, despite the fact that this protein has been involved in the expression of key regulators of the Caspase-dependent pathway in proliferating cells and in the immune system (Field, Tsai et al. 1996; DeGregori, Leone et al. 1997; Garcia, Murga et al. 2000; Moroni, Hickman et al. 2001; Muller, Bracken et al. 2001; Furukawa, Nishimura et al. 2002; Nahle, Polakoff et al. 2002; Pediconi, Ianari et al. 2003; Cao, Xia et al. 2004; Xie, Jiang et al. 2006; Lazzerini Denchi and Helin, 2005). The lack of effects on the expression of these genes in E2F1 and 2 double null mice is unlikely due to compensation by other members of the E2F1 family because E2F1 has been described to be the only member able of inducing apoptotic gene expression (Garcia et al., 2000), and because the ability of other E2F proteins for inducing apoptosis rely on endogenous E2F1 expression (Lazzerini Denchi and Helin, 2005).

If the key regulators of the Caspase-dependent cell death pathway are silenced in postmitotic tissues, the suggested involvement of Caspases in cardiac and neuronal diseases in the adult (Hermel et al., 2004; Hoglinger et al., 2007; Nam et al., 2004) could depend on the re-expression of the apoptotic signaling cascade in certain pathological conditions. The results presented here suggest that cultured primary adult cardiomyocytes can not re-express the apoptotic genes *in vitro* when submitted to certain death stimuli or E2F overexpression, although this does not rule out the re-expression of apoptotic genes *in vivo*. In neurons, expression of Apaf-1 has been reported to be enhanced in a model of brain injury (Yakovlev et al., 2001) and Caspase-2 upregulation occurs in certain neurons of a Huntington's disease mouse model (Hermel et al., 2004). However, experiments conducted to identify the mechanisms involved in neuronal and cardiac cell death during disease have been performed usually in embryonic cells. Our results put forward a note of caution on the interpretation of results obtained with embryonic cells or cell lines. We suggest the need to prove the implication of Caspases in adult cells to confirm the apoptotic gene re-expression event, which would have important clinical consequences.

PTBs increase translation of the major apoptotic genes probably involving internal ribosome entry site (IRES)

Our results show that PTB1 and PTB4 are expressed in the murine myocardium during embryonic cardiac development and are down-regulated progressively after birth, after a temporal pattern similar to that of apoptotic gene expression. Restoring PTB expression in postnatal cardiomyocytes was sufficient to increase apoptotic protein expression, probably involving IRES-dependent translation, and to induce a shift from Caspase-independent to Caspase-dependent DNA damage during experimental ischemia, which is Caspase independent in wild-type postnatal cardiomyocytes. To our knowledge, this is the first report on the mechanisms of regulation of apoptotic gene expression in the developing heart.

Participation of the apoptotic signaling in heart development (Lakhani et al., 2006; Yeh et al., 2000; Yeh et al., 1998) and silencing of this pathway during cardiomyocyte terminal differentiation *in vivo* (Bahi et al., 2006; Zhang et al., 2007a) and *in vitro* (Doss et al., 2007), suggest its strict regulation during development in

the myocardium. However, the mechanisms regulating apoptotic gene expression during cardiac development are unknown. Indeed, although gain of function or inactivation of many genes have been reported to induce alteration of the normal rate of cardiomyocyte apoptosis in the developing heart, the possible involvement of changes in the expression of apoptotic genes in these phenotypes was not assessed.

Despite the involvement of PTB proteins in alternative splicing of several genes relevant for cardiomyocyte function such as a-actinin (Southby et al., 1999), α-Tropomyosin (Patton et al., 1991), β-Tropomyosin (Mulligan et al., 1992), and Troponin-T (Charlet et al., 2002), as well as the role of PTB in the control of IRES dependent translation of several genes including pro-apoptotic Apaf-1 (Mitchell et al., 2003), there is no information about PTB expression and activity neither in mammalian cardiomyocytes nor in heart development. PTB has been suggested to be abundant in the adult chicken heart (Ladd et al., 2005), yet our results show that both rat and mouse myocardium spontaneously silence PTB expression early after birth.

Our observation that PTB1 and PTB4 are abundant in the developing embryonic heart and are silenced postnatally suggested to us their role in the control of gene expression during cardiomyocyte differentiation, in a similar way to what has been described in neurons (Boutz et al., 2007b; Makeyev et al., 2007) and in differentiating C2C12 myoblasts *in vitro* (Boutz et al., 2007a). PTB gain-of-function experiments presented here strongly suggest that PTB contribute to the posttranscriptional regulation of apoptotic gene expression and, hence, susceptibility to apoptosis in differentiating cardiomyocytes. Interestingly, the expression of the antiapoptotic gene Bcl-XL, unaffected by cardiomyocyte differentiation (Zhang et al., 2007a), was not modified by PTB. We wanted to identify the mechanisms mediating apoptotic gene expression driven by PTB in cardiomyocytes. PTB has been shown to contribute to Apaf-1 IRES-mediated translation in cell lines (Mitchell et al., 2001; Mitchell et al., 2003). To show this, authors used bicistronic vectors in which the Renilla Firefly (LucR) ORF and the Firefly Luciferase (LucF) ORF are separated by the 5'-UTR of Apaf-1. In transfected cells, LucR activity was a measure of transcription and cap-dependent translation and LucF activity required IRES-dependent translation, mediated by PTB in that case. The results presented here using similar bicistronic Luciferase reporter gene constructs suggest that PTB can

mediate IRES-dependent translation of Apaf-1 and Caspase-3 mRNAs in cardiomyocytes.

We also aimed at analyzing apoptotic gene expression in cardiomyocytes with PTB expression restricted to the cytosol in an attempt to exclude their nuclear functions (i.e. mRNA splicing). We transduced postnatal cardiomyocytes, which express low levels of endogenous PTB, with several PTB constructs harboring mutations or small deletions of amino acids of the N-terminus, reported to be relevant for nuclear targeting in cell lines (Perez et al., 1997; Romanelli et al., 1997). However, neither single mutation nor mutation of all key residues altered significantly PTB targeting to the nucleus. This suggested that other regions in PTB are important for nuclear expression in primary cardiomyocytes. We decided to delete both the NLS and first RRM because RRM1 has been shown to be important for nuclear localization (Kamath et al., 2001; Perez et al., 1997) and for protein–protein interactions (Kim et al., 2000) yet dispensable for PTB–RNA interactions (Perez et al., 1997). This short PTB construct conserved the primary determinant for RNA binding specificity, which resides in RRM3 and RRM4, as shown in Vero and HeLa cell lines (Oh et al., 1998; Perez et al., 1997), and the primary contributor to dimer stabilization, which resides in RRM2 (Oh et al., 1998; Perez et al., 1997). Unexpectedly, transduction of PTB lacking the NLS and RRM1 in cardiomyocytes induced PTB expression in the cytosol at some extent, yet most of the exogenous PTB was still directed to the nucleus and induced nPTB exon 10 skipping. Nuclear localization of PTB lacking the NLS and RRM1 in primary cardiomyocytes was in apparent contradiction with earlier reports. Indeed, PTB-GST constructs lacking the NLS and RRM1 had been reported to be expressed exclusively in the cytosol of Vero monkey cells (Perez et al., 1997), whereas fussing an N-terminus PTB fragment to CAT was sufficient to direct its expression to the cytosol in 3T3 cells, and deletion of GTK or KKF motifs of the N-terminal region restricted CAT expression to the cytosol (Romanelli et al., 1997). One difference between this study and other's reports, in addition to the use of primary cells instead of cell lines, is that we have analyzed PTB distribution with anti-PTB antibodies and not by detection of a long tag fussed to the protein or a PTB fragment. Our approach implies the possibility of detecting short PTB-induced expression of endogenous full-length PTB that would be directed to the nucleus. However, this was discarded by western blot analysis

showing no changes in endogenous PTB expression in these experiments. Finally, abnormal behavior of PTB distribution due to overexpression in cardiomyocytes that does not happen in overexpressing cell lines is improbable because, in our hands, overexpression of other genes using identical procedure did not alter their expected subcellular distribution ((Bahi et al., 2006) and data not shown). Thus, these results suggest that other regions outside the NLS and RRM1 of PTB are relevant for its intracellular localization in cardiomyocytes and/or that PTB can associate with other proteins to enter the nucleus without requiring the NLS. These results also showed that NLS and RRM1 of PTB are important for inducing expression of a subset of apoptotic genes (e.g. Caspase-9, Bax, and Bid), but not for the regulation of other target genes (e.g. Apaf-1 and Caspase-3).

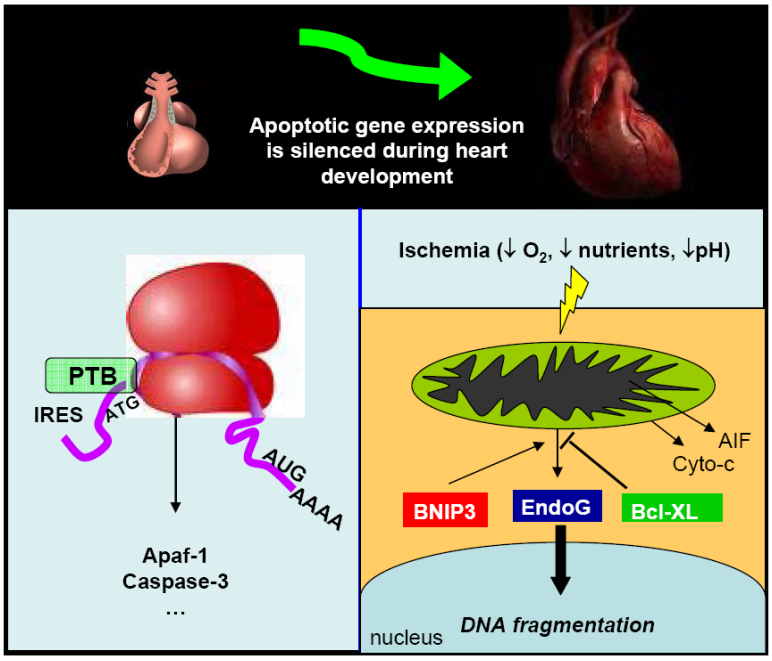


Figure 23. Model showing the regulation of apoptotic gene expression by PTB through IRES-dependent translation in cardiomyocytes during development and the Caspase-independent cell death signaling induced by ischemia in differentiated cardiomyocytes, involving EndoG, BNip3 and Bcl-XL.

Our results also show that the slight increase in apoptotic gene expression induced by PTB, probably in addition to modifications in the expression of other target genes relevant for apoptosis, is sufficient for changing the main pathway leading to cardiomyocyte damage during stress (i.e. from Caspase-independent DNA

damage to Caspase activation and Caspase-dependent DNA damage). These results show that small changes in apoptotic gene expression modify the susceptibility of cardiomyocytes to apoptotic stimuli, and point to PTB as relevant regulators in the control of the susceptibility of differentiating cardiomyocytes to Caspase-dependent apoptosis through posttranscriptional regulation of apoptotic gene expression, involving IRES-dependent translation.

In summary, we have found that the key regulators of the canonical caspase-dependent apoptosis pathway are silenced during heart development. This silencing correlates with the switch of Caspase-dependent cell death to Caspase-independent cell death in cardiomyocytes. Release of EndoG from mitochondria is a key event of Caspase-independent cell damage related with different stress stimuli in neonatal cardiomyocytes. BNip3 induces and Bcl-XL can block the ischemia induced release of EndoG from mitochondria. Once released from mitochondria, EndoG contributes to the Caspase-independent DNA damage. E2Fs have no effect in controlling the developmental downregulation of apoptotic proteins contrary to what has been observed by others in tumor cells. Polypyrimidine Tract binding protein (PTB) can regulate expression of apoptotic proteins by inducing their translation, probably through IRES-dependent mechanism without affecting the transcription level (Figure 23).

Conclusions

1. Expression of the genes implicated in the caspase-dependent signaling is silenced during postnatal development, notably in the heart and the brain.

2. Ischemia and Staurosporin-induced DNA damage is caspase-dependent in embryonic cardiomyocytes, but occurs through caspase-independent mechanisms in postmitotic cardiomyocytes.

3. EndoG is the main contributor of ischemia-induced DNA fragmentation in postmitotic cardiomyocytes.

4. BNip3, a BH3-only Bcl-2 family protein, controls ischemia-induced DNA fragmentation in postmitotic cardiomyocytes through promoting the release of EndoG from mitochondria. On the contrary, Bcl-XL, an antiapoptotic Bcl-2 family protein, blocks the release of EndoG from mitochondria and can inhibit DNA fragmentation in postmitotic cardiomyocytes.

5. E2F1and E2F2 are neither involved in apoptotic gene expression in the embryo nor does its development-linked repression affect apoptotic gene expression in the adult heart.

6. The main regulators of the caspase-dependent cell death are not re-expressed in cultured adult cardiomyocytes treated with stress stimuli or E2F1 overexpression.

7. Polypyrimidine Tract Binding Proteins (PTBs), which are expressed in the embryonic heart and are silenced during cardiomyocyte differentiation, can upregulate apoptotic gene expression in differentiated cardiomyocytes and induce a switch from caspase-independent to caspase-dependent DNA damage during experimental ischemia.

8. PTBs regulate apoptotic gene expression in postnatal cardiomyocytes through post-transcriptional mechanisms probably involving IRES-dependent translation.

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Annex

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