L'expression de BARD1 et sa fonction potentielle dans le cancer = (Expression of BARD1 isoforms and possible function in cancer)

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L'expression de BARD1 et sa fonction potentielle dans le cancer

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Thèse

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par

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Dedicated to

my parents, my husband and my young daughter,

who give me their deepest love and great supports.

Expression of BARD1 isoforms and possible function in cancer

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List of abbreviations

AJCC	American Joint Committee on Cancer
ANK	Ankyrin domain
BARD1	BRCA1-associated RING domain protein
BRCT	BRCA1 carboxy-terminal repeat
CTB	Cytotrophoblast
DAB	Diaminobenzidine tetrahydrocholoride
DSBs	Double strand breaks
EtB	Ethidium bromide
FL	Full length
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
HMGB1	High mobility group protein 1
hCG	Human chorionic gonadotropin
MMP	Matrix metalloproteinases
MW	Molecular weight
NES	Nuclear export signal
NLS	Nuclear locating signal
PMA	Phorbol 12-myristate 13-acetate
RING	RING (really interesting new gene) domain
RT-PCR	Reverse transcription polymerase chain reaction

Résumé

Le suppresseur de tumeur BARD1 a été initialement découvert comme partenaire protéique de BRCA1. Les protéines BARD1 et BRCA1 forment un hétérodimère stable qui présente une activité ubiquitine-ligase E3. Cet héterodimère est impliqué dans plusieurs fonctions onco-suppressives. Par ailleurs, la protéine BARD1 présente des fonctions indépendantes de BRCA1 tel que son rôle dans l'apoptose ou dans le contrôle de l'homéostasie tissulaire.

Peu de mutations du gène BARD1 ont été retrouvées dans les cancers. Cependant, la surexpression de protéines BARD1 tronquées a été observée dans des cancers du sein et de l'ovaire et est associée à un pronostique sévère. Ceci nous a conduit à étudier l'expression des isoformes de BARD1 dans des cellules cytotrophoblastiques et dans différents types de cancers. Nous avons ainsi montré que les cytotrophoblastes humains, qui présentent une forte similarité avec des cellules cancéreuses de part leur comportement invasif, surexpriment des formes tronquées de BARD1 produites par épissage alternatif. Par ailleurs, l'expression de ces isoformes est régulée par l'hormone gonadotrophine chorionique humaine et par hypoxie. Plus particulièrement, l'isoforme δ est exprimée dans les villosités et les CTBs en prolifération alors que l'isoforme ε est exprimée dans les CTBs invasifs. De manière intéressante, nous avons observé que les isoformes de BARD1 sont sécrétées par les cytotrophoblastes, sécrétion associée à des défauts d'adhésion de cellules épithéliales en culture. Ainsi, les isoformes de BARD1 pourraient être impliquées dans l'invasion trophoblastique. Dans différentes lignées de cellules cancéreuses, nous avons observé une expression différentielle des isoformes ainsi qu'une réinitiation transcriptionnelle dans l'exon 4. De plus, les isoformes sont beaucoup plus fortement exprimées que la forme entière de BARD1. Par marquage immunohistochimique, nous avons observé une expression différencielle des isoformes de BARD1 en fonction du stade et du type histologique de cancer de l'ovaire. En effet, les isoformes δ et ε sont très fortement exprimées dans les cancers ovariens les plus agressifs. Ainsi, certaines isoformes de BARD1 pourraient constituer des marqueurs de pronostique de cancer de l'ovaire.

Introduction

La protéine BARD1 (BRCA1-associated RING domain 1) a été initialement découverte en tant que partenaire de la protéine BRCA1. Cette protéine fait l'objet d'études de plus en plus nombreuses visant à mieux comprendre son rôle, dépendant ou non de BRCA1, dans la tumorigenèse.

1. Structure de la protéine BARD1

Le gène BARD1, localisé sur le chromosome 2 en position 2q34-q35, s'étend sur une région de 10kb proche du télomère. Ce gène est composé de 11 exons et code une protéine de 777, 765 et 768 acides aminés chez l'homme, la souris et le rat respectivement. La protéine BARD1 ne présente pas de similarité de séquence ou de structure avec BRCA2 par contre sa structure est très proche de BRCA1. En effet, BARD1 et BRCA1 comportent toutes deux un domaine RING finger dans leur région N-terminale et deux domaines BRCT à leur extrémité C-terminale. La protéine BARD1 possède également trois motifs ankyrine. Ces domaines sont conservés au cours de l'évolution ce qui n'est pas le cas des régions centrales de ces protéines. Par ailleurs, six signaux de localisation nucléaire ou NLS ont été prédits dans la séquence de BARD1.

2. Expression de BARD1 et localisation cellulaire

Dans la plupart des tissus murins, BARD1 et BRCA1 présentent une expression concomitantes. Ainsi, il a été montré que BARD1, tout comme BRCA1, est fortement exprimée dans la rate, les testicules et dans les tissus prolifératifs. Les transcripts codant ces deux protéines sont également co-exprimés dans la glande mammaire et dans l'embryon

murin. Cependant, BARD1 présente une expression indépendante de BRCA1 dans les organes hormono-dépendants comme l'utérus.

La protéine BARD1 a été décrite comme une protéine nucléaire. En effet, il a été montré que BARD1 colocalise avec BRCA1 et la protéine de réparation RAD51 au niveau de foci nucléaires durant la phase S du cycle cellulaire ou suite à des dommages de l'ADN. D'autre part, BARD1 est impliquée dans l'import nucléaire de BRCA1 mais également dans sa rétention dans le noyau. En effet, BARD1 en se fixant au domaine RING finger de BRCA1 masquerait les deux séquences d'export nucléaire de BRCA1, nécessaire à sa translocation cytoplasmique.

Certaines études ont montré que BARD1 pouvait être localisé dans le cytoplasme, ce qui stimulerait son activité apoptotique. Par ailleurs, des formes tronquées de BARD1 sont fortement exprimées dans les cancers du sein et de l'ovaire et présentent une localisation cytoplasmique.

3. Fonction biologique de BARD1

La protéine onco-suppressive BARD1 est impliquée dans le réparation de l'ADN, la régulation transcriptionnelle, la maturation des ARN, l'ubiquitinylation, l'apoptose et la mitose. En tant que partenaire de BRCA1, BARD1 participe au maintien de l'intégrité du génome. Ainsi, l'inhibition de l'expression de BARD1 entraîne une instabilité génétique, une perte de polarité des cellules et une létalité embryonnaire dans les souris knock-out.

3.1. BARD1/BRCA1, un complexe à activité ubiquitine ligase

L'ubiquitinylation est l'une des fonctions majeures du complexe BARD1/BRCA1. En effet, il a été montré que les domaines RING finger de BARD1 et BRCA1 leur confèrent une activité ubiquitine-ligase E3. In vitro, BRCA1 et BARD1 présentent individuellement une très faible

activité ubiquitine-ligase, activité qui devient forte lorsque ces deux protéines forment un complexe. Le complexe BRCA1/BARD1 est capable de s'autoubiquitinyler, ce qui stimule sa propre activité. Il peut également ubiquitinyler *in vitro* la protéine H2AX, qui colocalise avec BRCA1 au niveau de sites de cassures de l'ADN, ainsi que la tubuline γ, principal composant des centrosomes. L'ubiquitinylation de la tubuline γ par BARD1/BRCA1 serait impliquée dans la régulation du nombre de centrososmes et de leur activité.

L'activité ubiquitine-ligase du complexe BARD1/BRCA1 a été décrite plusieurs fois *in vitro*, et plus récemment dans les cellules de mammifères. Il a ainsi été montré que ce complexe est associé au processus d'ubiquitinylation au niveau de foci nucléaires en phase S, mais également au niveau des cassures de l'ADN suite à l'induction de dommages. Le complexe BARD1/BRCA1 est capable d'ubiquitinyler la forme phosphorylée de l'ARN polymérase II, cible identifiée *in vitro* et *in vivo* suite à l'induction de dommages dans l'ADN. En effet, l'inhibition de BRCA1 et de BARD1 par interférence à l'ARN entraîne la stabilisation de l'ARN polymérase II après dommages de l'ADN. Ces résultats confortent un modèle selon lequel suite à des dommages de l'ADN, le complexe BARD1/BRCA1 permettrait la dégradation de l'ARN polymerase II au niveau des sites de cassures, empêchant la transcription des brins endommagés et facilitant ainsi leur réparation.

L'ajout de chaînes d'ubiquitines à une protéine cible ne conduit pas forcément à sa dégradation. Cela dépend notamment de la position de la lysine de l'ubiquitine, qui sert de site d'attachement à la protéine cible. Or plusieurs études ont montré que l'hétérodimère BRCA1/BARD1 catalyse l'ajout de chaînes d'ubiquitines par leurs lysines 6 aux protéines cible, processus qui ne conduit pas nécessairement à la dégradation protéique contrairement à la fixation d'ubiquitines par d'autres lysines comme la lysine 48. Ainsi, la protéine nucleophosmin/B23, impliquée dans plusieurs processus nucléaires comme le remodelage de la chromatine, a été identifiée comme substrat de l'activité ubiquitine-ligase de

BRCA1/BARD1 *in vivo*. L'ubiquitinylation de cette protéine, dont la colocalisation avec le complexe BRCA1/BARD1 a été observée pendant la mitose, entraîne sa stabilisation et non sa dégradation.

La régulation de l'activité du complexe BRCA1/BARD1 est peu connue. Il a récemment été montré que les protéines CDK2 (Cyclin Dependent Kinase 2) et cycline E1, en s'associant au complexe BRCA1/BARD1, entraînent sa déstabilisation, corrélée à l'export nucléaire de BRCA1 et de BARD1.

Le rôle cellulaire du complexe enzymatique BRCA1/BARD1 est encore mal connu. Peu de substrats ont été identifiés mais permettent néanmoins d'impliquer l'activité ubiquitine-ligase de ce complexe dans différents processus cellulaires comme la réponse aux dommages de l'ADN.

3.2. Fonctions de BARD1 indépendantes de BRCA1

BARD1 présente également des fonctions indépendantes de BRCA1. Elle est notamment impliquée dans l'apoptose dépendante de p53. L'interaction entre BARD1 et p53 facilite la phosphorylation de p53 et sa stabilisation. Il a ainsi été montré qu'en absence de protéines BARD1 fonctionnelles, la phosphorylation de la sérine 15 de p53 est inhibée. BARD1 se lie à la protéine Ku-70, sous-unité de la kinase DNA-PK, et catalyse ainsi la phosphorylation de p53.

D'autre part, il a été montré que BARD1 interagit, indépendamment de BRCA1, avec le facteur CstF-50, impliqué dans le clivage endonucléolytique de l'ARN, ainsi qu'avec les facteurs transcriptionnels NFκB/Rel. Ceci suggère un rôle de BARD1 dans le contrôle de la prolifération cellulaire et dans la régulation de la transcription.

4. BARD1 et cancer

Des mutations des gènes BRCA1 et BRCA2 sont présentes la plupart des cas familiaux de cancer du sein et/ou de l'ovaire. BARD1 est tout comme BRCA1 et BRCA2 un gène suppresseur de tumeur, néanmoins il est rarement muté dans les cancers. En effet, l'analyse de nombreux cancers sporadiques du sein et de l'ovaire n'a permis de mettre en évidence que trois mutations faux-sens, Q564H, V695L et S761N. Une perte d'hétérozygotie a été observée dans les cancers associés à deux de ces mutations.

De manière intéressante, beaucoup de mutations de BRCA1 affectent son domaine RING finger, abolissant ainsi l'activité du complexe BARD1/BRCA1, alors que les mutations affectant le gène BARD1 affectent les motifs ankyrine, le domaine BRCT ou la région séparant ces domaines.

Par ailleurs, des études d 'immunohistochimie ont montré de manière inattendue que BARD1 est fortement exprimée dans les cellules tumorales et présente une localisation cytoplasmique. Cette forte expression pourrait correspondre à celle d'isoformes de BARD1 produites par épissage alternatif. En effet, une isoforme de BARD1 issue de l'épissage alternatif des exons 2 à 6 a été identifiée dans des cellules cancéreuses ovariennes de rat puis retrouvée dans la lignée cancéreuse humaine HeLa.

Ainsi, afin de comprendre le rôle de BARD1 dans la cancérogenèse, nous nous intéressons à l'expression de BARD1 et de ses isoformes dans les cancers et à son implication dans la croissance des cellules cancéreuses.

Abstract

The tumour suppressor BARD1, originally discovered as BRCA1-binding protein, acts in conjunction with BRCA1 as ubiquitin ligase. BARD1 and BRCA1 form a stable heterodimer and dimerization, which is required for tumour suppressor functions attributed to BRCA1. In addition, BARD1 has BRCA1-independent functions in apoptosis, and a role in control of tissue homeostasis was suggested. However, cancer-associated mutations of BARD1 are rare, on the contrary, overexpression of truncated BARD1 isoforms was found in breast and ovarian cancer and correlated with poor prognosis. We performed experiments to elucidate BARD1 isoform expression in cytotrophoblasts, and in various cancer cells. Here we report that human cytotrophoblasts, which show a strong similarity with cancer cells in respect to their invasive behavior, overexpress truncated forms of BARD1 derived from differential splicing, and expression of these isoforms is regulated by human chorionic gonadotropin and by hypoxia. In particular, we found isoform δ expressed in villous and proliferative cytotrophoblasts, and isoform ε in invasive cytotrophoblasts. Interestingly, we identified isoforms of BARD1 that were secreted by cytotrophoblasts interfered with the adhesion of epithelial cells in culture, suggesting that BARD1 isoforms might have a function in cytotrophoblasts invasion. In cancers cells we observed the same isoforms derived from differential splicing as in cytotrophoblasts. And in addition, we found new transcriptional initiations in exon 4, and a specific signature of BARD1 isoform expression pattern in cancer cell lines from different origin. Compared to full length BARD1, BARD1 isoform expression is upregulated in cancer cells. Applying immunohistochemistry on a collection of ovarian cancers, we found that loss of the N-terminal region of BARD1 is correlated with advanced stage of cancer and expression of spliced isoforms is typical for clear cell ovarian carcinoma. Therefore, specific isoforms of BARD1 might be a negative prognostic factor in ovarian

cancer. We further hypothesize that BARD1 isoforms might play a functional role in cancer development and progression.

Introduction

The BRCA1-associated RING domain protein 1 (BARD1) was originally discovered as a protein interacting with BRCA1 to which it is structurally related. BARD1 has been attracting more and more attention in the last few years, both as binding partner of the breast cancer protein BRCA1 and independently due to its potential function in tumorigenesis.

1. Structure of BARD1

It has been over a decade since BARD1 was first identified in a yeast two-hybrid screen as interacting protein of BRCA1 (Wu et al., 1996). The BARD1 gene spans a 10 kb region close to the telomere on chromosome 2q34-q35. The BARD1 gene is composed of 11 exons, which encode a protein of 777 (Wu et al., 1996), 765 (Ayi et al., 1998), or 768 (Gautier et al., 2000) amino acids in human, mouse, and rat, respectively. BARD1 has no sequence or structural similarity with BRCA2, but it shares a homologous structure with BRCA1 (Fig. 1). The BRCA1 gene is composed of 24 exons and encodes 1863 amino acids (Miki et al., 1994). BARD1 and BRCA1 share structural similarity in conserved N-terminal RING finger and two C-terminal BRCT domains. This suggests that both proteins are derived from a common ancestor that comprised RING domain and BRCT domain (Irminger-Finger and Jefford, 2006; Irminger-Finger and Leung, 2002). BARD1 and BRCA1 genes have been found in several species: mouse (Ayi et al., 1998; Irminger-Finger et al., 1998), rat (Gautier et al., 2000), Xenopus (Joukov et al., 2001), C. elegans (Boulton et al., 2004), and a database entry is found for the tropic fish Takifugu rubripes. While the N-terminal RING finger and the BRCT domains of BARD1 and BRCA1 are evolutionary conserved with at least 90% identity of amino acids, the regions between these structures show only little conservation (Irminger-Finger et al., 1998). In addition, BARD1 possesses three internal tandem ankyrin (ANK)

repeats, which are highly conserved (Ayi et al., 1998; Gautier et al., 2000; Irminger-Finger et al., 1998) (Fig. 1). These three highly conserved structural domains might mediate essential functions for BARD1.

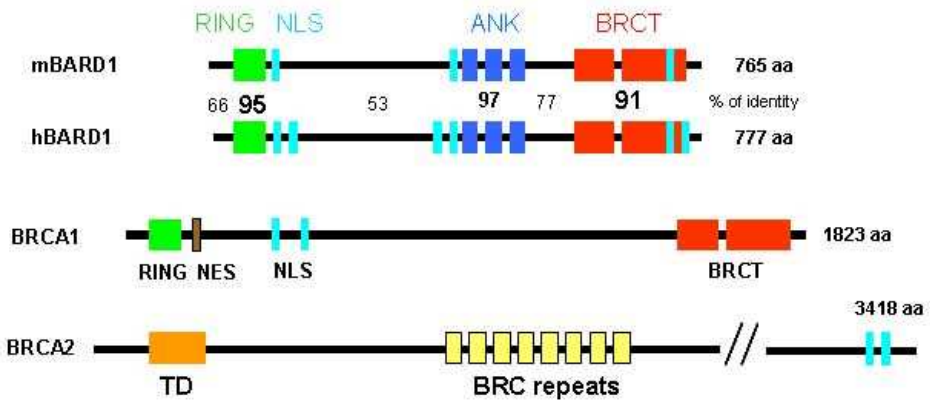


Figure 1. **Schematic presentation of BARD1, BRCA1 and BRCA2 proteins showing conserved functional domains.** BARD1 and BRCA1 are homologous in sharing two conserved domains (RING and BRCT), whereas BRCA2 is completely unrelated to either BARD1 or BRCA1 with conserved transcription activation domain (TD) and BRC repeats. Similarity of human and mouse BARD1 is shown as percentages. Ankyrin repeats (ANK), nuclear localization signals (NLS, light blue) and nuclear export signal (NES) are indicated.

BARD1 and BRCA1 form a heterodimer via their RING finger domains which are critical for the proper association of the two proteins (Meza et al., 1999; Wu et al., 1996). The RING finger motifs correspond to residues 24-64 and residues 50-86 in BRCA1 and BARD1, respectively. The BRCA1 RING motif is characterized by a short antiparellel three-strand β-sheet, two large Zn2+ binding loops and a central α-helix. The BARD1 RING motif is structurally homologous but lacks a central helix between the pair of third and fourth Zn2+ ligands. BARD1 is five residues shorter than BRCA1 within this segment (Brzovic et al., 2001b). Although the BRCA1 and BARD1 RING motifs are juxtaposed in the heterodimer, they do not pack tightly against each other. *In vitro* studies showed that individually BRCA1 and BARD1 exist as homodimers, but they preferentially form heterodimers implicating residues 1–109 of BRCA1 and residues 26–119 of BARD1, which are more stable (Meza et al., 1999). In addition to the three functional domains, human BARD1 has 6 predicted nuclear

localization signals (NLS), situated in the vicinity or embedded in each of the three functional domains, inferring a predicted nuclear localization of BARD1 (Jefford et al., 2004).

The structures of the ANK repeats and BRCT domains are less well known. The tandem repeat detected at the C terminus of BRCA1 and BARD1, subsequently termed BRCT repeats, was also found in many repair proteins (Caldecott et al., 1995; Ljungquist et al., 1994). BRCT repeats are defined by conserved clusters of hydrophobic residues that occupy the core of the repeat structure and by glycine residues that facilitate a tight turn between α1 and β2. It is a basic fold of a single repeat consisting of a parallel four-stranded β-sheet, which is flanked on one side by a pair of α-helices (α1 and α3) and on the other side by a single α-helix (Glover et al., 2004). Truncation or complete loss of both BRCT repeats in BRCA1 are associated with cancer incidence, indicating that BRCT is also an essential region for tumour suppressor functions (Glover et al., 2004; Huyton et al., 2000; Williams and Glover, 2003).

Ankyrin domains are found in sets of repeats, usually of 3 or 4, but can be as many as 20 in proteins of various functions (Huyton et al., 2000; Mosavi et al., 2004). Their precise functions in BARD1 remain unclear.

2. Expression and cellular localization of BARD1

In most murine tissues, BARD1 and BRCA1 are concomitantly expressed (Irminger-Finger et al., 1998). Northern blot experiments showed that BARD1 RNA messengers were abundantly expressed in spleen and testis, but not in liver, lung, skeletal muscle, heart, brain or kidney (Ayi et al., 1998), and more sensitive RNase protection experiments showed expression of BARD1 in most proliferate tissues (Ayi et al., 1998; Irminger-Finger et al., 1998). BARD1 and BRCA1 transcripts are also co-ordinately expressed in the mammary gland and in the mouse embryo (Irminger-Finger et al., 1998).

However, expression of BARD1 and BRCA1 was non-coordinate in hormonally controlled organs. In the uterus, BARD1 expression increased from di-oestrus through post-oestrus phase, whereas BRCA1 increases from diestrus to early oestrus and decreases during oestrus and post-oestrus (Irminger-Finger et al., 1998). In testis, BARD1 is expressed at all stages of spermatocyte maturation, whereas BRCA1 expression is only seen in meiotic and early round spermatocytes (Feki et al., 2004; Scully et al., 1997b).

BARD1 was originally found in nuclear extracts and described as a nuclear protein (Wu et al., 1996). Concomitant expression of BARD1 and BRCA1 was observed during S phase (Hayami et al., 2005). Indeed, BARD1 colocalized with BRCA1 and repair protein Rad51 in nuclear dots during S phase *in vivo* (Jin et al., 1997), and to nuclear foci in response to DNA damage (Scully et al., 1997a). A mutation in the RING finger of BRCA1, disrupting BRCA1-BARD1 interactions, abolished the formation of nuclear foci (Chiba and Parvin, 2002; Fabbro et al., 2002), indicating that this region is necessary for BARD1-BRCA1 colocalization. Further studies show that BARD1 can play a chaperone role in correct translocation of BRCA1 into nucleus (Fabbro et al., 2002). BARD1 retains BRCA1 in the nucleus by masking BRCA1's NES (Nuclear Export Signal) sequence (Brzovic et al., 2001a; Fabbro et al., 2002; Schuchner et al., 2005). Therefore, BARD1 plays an important role in trapping BRCA1 within the nucleus. Thus, the concomitant expression of BARD1 and BRCA1 supports the functions ascribed to the BRCA1-BARD1 heterodimer.

Further studies found that BARD1 can be also expressed in cytoplasm. Its cytoplasmic localization is associated with apoptosis function. These observations suggest that BARD1 can shuttle from the nucleus to the cytoplasm, correlated with its apoptotic activity (Jefford et al., 2004; Rodriguez et al., 2004).

Interestingly, aberrantly elevated expression of a truncated BARD1 which presents a cytoplasmic localization, was found in breast and ovarian cancers, and the level of expression of truncated BARD1 was correlated with poor prognosis (Wu et al., 2006).

3. Biological function of BARD1

BARD1 is regarded as a tumour suppressor and plays an important role in normal proliferation and tumour suppression. Loss of BARD1 may result in early embryonic lethality and chromosomal instability (McCarthy et al., 2003). BARD1-repressed cells show a prolonged S-phase, genetic instability, loss of growth inhibition by contact, and loss of morphogenetic properties (Irminger-Finger et al., 1998). A high number of proteins interact or associate with BARD1 (Figure 2). BARD1 may, through BRCA1 or independently regulate, chaperone and serve as a scaffold for numerous proteins involved in a number of cellular pathways ranging from DNA repair, transcriptional regulation, apoptosis, genomic integrity and mitotic events (Jasin, 2002).

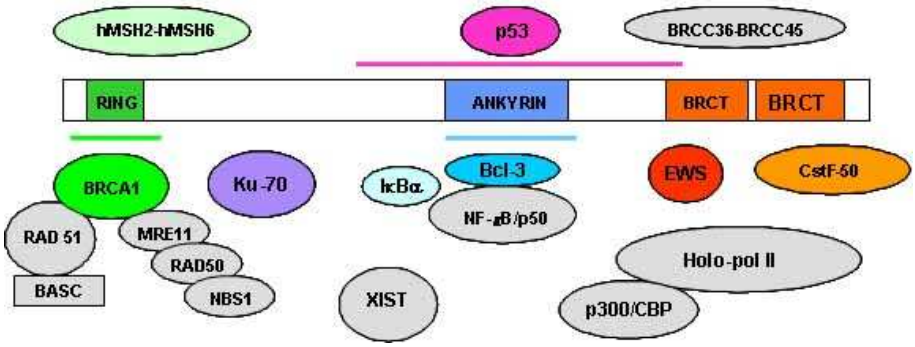


Figure 2. **Illustration showing the known proteins that interact with BARD1.** Proteins that are thought to have a direct interaction are shown in colour. Proteins that are known to bind indirectly with BARD1 are shown in grey, all of which may be detected in biochemical complexes.

3.1. The functions of the BRCA1-BARD1 heterodimer

As a coordinator with BRCA1, BARD1 plays an important role in maintaining genomic stability and phenotype. Dissection of repair pathways showed that the BRCA1-BARD1 heterodimer has a role in homologous repair before the branch point of HDR (homology derived repair) and SSA (single strand annealing) (Stark et al., 2004). In response to DNA damage, BARD1 and BRCA1 colocalize with proliferating cell nuclear antigen (PCNA), a protein involved in DNA replication (Cox., 1997) and with Rad51 (Jin et al., 1997; Scully et al., 1997b), a protein involved in eucaryotic double strand break repair (Shinohara et al., 1992). This dynamic localization is consistent with a role for BRCA1-BARD1 complexes in DNA replication checkpoint response (Gowen et al., 1998).

One of the most important mechanisms of BRCA1-BARD1 function is the ubiquitin ligase activity. Ubiquitin ligases are enzymes that ligate the small protein ubiquitin to other target proteins, which are then recognized by the proteasome and delivered for degradation. Now it is widely accepted that the N-terminal RING domain of BRCA1 and BARD1 confers E3 ubiquitin-ligase activity, which targets proteins involved in cell cycle regulation and DNA repair (Baer and Ludwig, 2002). The importance of this enzymatic activity is highlighted by observations that tumour-associated mutations C61G and 64G which lie within the RING domain of BRCA1 are defective in E3 ubiquitin-ligase activity (Brzovic et al., 2001a; Hashizume et al., 2001; Ruffner et al., 2001). This demonstrates that E3 ubiquitin-ligase activity is critical for BRCA1-BARD1 functions and suppression of tumorigenesis.

In vitro, BRCA1 and BARD1 have very low ubiquitin ligase activity individually, but BRCA1's activity is significantly enhanced when bound to BARD1 (Hashizume et al., 2001). The results of mutagenesis studies indicate that the enhancement of BRCA1 E3 ligase activity depends in direct interaction of BARD1 and BRCA1 (Xia et al., 2003). This reflects that the heterodimer is a more stable structure. The BRCA1–BARD1 heterodimer directs

polymerization of ubiquitin primarily through K6, which is an unconventional linkage (Wu-Baer et al., 2003). BRCA1 and BARD1 also undergo auto-ubiquitylation, which results in 20-fold stimulation of E3 ligase activity in vitro (Chen et al., 2002; Mallery et al., 2002). The autoubiquitination reactions promote formation of poly-linked ubiquitin chains attached at K6 (Nishikawa et al., 2004; Wu-Baer et al., 2003). The ubiquitinated BRCA1-BARD1 complex has an increased affinity for binding to DNA repair intermediates (Simons et al., 2006), suggesting that this modification is a regulator of BARD1-BRCA1 activity in DNA damage response.

Some of the first identified targets of the BRCA1-BARD1 ubiquitin ligase function were the histones H2A and H2AX (Ruffner et al., 2001). Attachment of a single ubiquitin to histones H2A and H2B leads to alternation of chromatin structure and opens DNA for transcriptional activity (Davie and Murphy, 1990; Levinger and Varshavsky, 1982), indicating a role for BRCA1-BARD1 in transcriptional activation. BRCA1-BARD1 has been shown to ubiquitinate phosphorylated RNA polymerase II (RNA Pol II) complex as part of a possible genome surveillance pathway (Kleiman et al., 2005; Starita et al., 2005). The BRCA1-BARD1 complex ubiquitinates the phosphorylated RNA Pol II in response to DNA damage. Depletion of BRCA1 and BARD1 in cells by siRNA treatment significantly reduced ubiquitination of RNA Pol II after DNA damage (Kleiman et al., 2005), implicating BARD1 and BRCA1 in controlling transcription activity. A more recent report suggests that BRCA1-BARD1 mediates polyubiquitination of RPB8, a common subunit of three types of RNA polymerases, in response to DNA damage, which indicates an important role of BARD1 for cell survival after DNA damage (Wu et al., 2007).

More data reveal that BRCA1-BARD1 has a role in preventing double strand breaks (DSBs) by regulating the activity of topoisomerase II α (topo II α) in an ubiquitination dependent manner (Lou et al., 2005). *In vivo*, BRCA1-BARD1 ubiquitination of topo II α stimulates its

activity, regulates the mobility of topo II α, and consequently DNA decatenation, which implies an important role for protecting cells from DNA damage.

Recently, light was shed on the role of BARD1 and BRCA1 in cell-cycle progression through ubiquitination processes. BRCA1-BARD1 has an important function in check-point control. The regulation of centrosome number is critical for mitosis. BRCA1-BARD1 ubiquitin ligase activity may directly regulate centrosome number, which is important for maintaining chromosomal stability and neuploidy (Lingle et al., 2002; Pihan et al., 2003). A number of centrosome proteins were found as targets of BRCA1-BARD1, and one of those was γ-tubulin. *In vivo*, BRCA1-BARD1 ubiquitylates γ-tubulin by using K48 and K344 residues (Starita et al., 2004). Another centrosome protein that is targeted by the BRCA1-BARD1 ubiquitin ligase is the nucleolar phosphoprotein nucleophosmin 1 (NPM1), which is an important regulator of chromosome stability (Grisendi et al., 2005). In human tumours, mutations of NPM are associated with haematological disorders. Therefore, the ubiquitylation function driven by BRCA1-BARD1 heterodimer is responsible for mediating checkpoint functions and cell cycle arrest.

A recent report identified estrogen recetor α (ERα) as a putative substrate for the BRCA1-BARD1 ubiquitin ligase (Eakin et al., 2007). The regions of BRCA1-BARD1 necessary for ERα ubiquitination include the RING domains and at least 241 and 170 residues of BRCA1 and BARD1, respectively. Cancer-predisposing mutations within this region abrogate ERα ubiquitination. It is well-known that endogenous exposure to female reproductive hormones is a central factor in the development of many cancers, such as breast (Conneely et al., 2003; Trauernicht and Boyer, 2003) and ovarian cancer (Sun et al., 2005). This report therefore suggests a link between BRCA1-BARD1 ligase activity and hormone dependent carcinoma.

In summary, it is reasonable to predict the role of BRCA1 and BARD1 in diverse cellular functions as DNA repair, transcription and checkpoint signalling by achieving through ubiquitylation of specific target proteins.

3.2. BRCA1-independent pro-apoptotic functions of BARD1

Although BARD1 and BRCA1 form a heterodimer *in vivo* and *in vitro*, it has been observed that BARD1 and BRCA1 were not consistently coexpressed in all tissues (Irminger-Finger et al., 1998). BARD1 and BRCA1 expression levels are modulated differently in hormonally controlled tissues during the ovulatory cycle of the mouse (Irminger-Finger et al., 1998) and spermatogenesis of rats (Feki et al., 2004), indicating BARD1 might have BRCA1 independent functions.

In vivo, BARD1 expression is absent in the central nervous system, but it was upregulated in response to hypoxia, whereas BRCA1 was not detected (Irminger-Finger et al., 2001). *In vitro*, BARD1 upregulation upon genotoxic stress is transcriptionally regulated. Elevated expression of BARD1 was associated with apoptosis. Additionally, overexpression of exogenous BARD1 leads to DNA fragmentation and caspase-3 activation, indicative of apoptosis. Transduction of BARD1 in BARD1 deletion mutant induces apoptosis, but overexpression of BRCA1 does not have the same effect. On the contrary, transfection of BRCA1 diminished rather than enhanced apoptosis induction by BARD1 (Irminger-Finger et al., 2001).

Furthermore, BARD1 exerts its action on p53 at a post-transcriptional level by binding to and stabilizing of P53. It was found that the increased BARD1 expression level is accompanied by an increase in p53 protein levels but not mRNA levels (Irminger-Finger et al., 2001). BARD1 is implicated in stabilization and phosphorylation of p53, since the absence of functional BARD1 is sufficient for abolishing p53 phosphorylation on serine 15 (Fabbro et al., 2004).

Indeed, BARD1 binds to Ku-70, a subunit of DNA-dependent protein kinase (DNA-PK) and catalyzes phosphorylation of p53 (Feki et al., 2005). The minimal required binding region for p53 binding and p53-dependent apoptosis in BARD1 is residue 510-604 which is between ANK and BRCT domains. C557s and Q564H are two known cancer predisposing mutations that localize to this region (Ghimenti et al., 2002; Jefford et al., 2004; Karppinen et al., 2004; Thai et al., 1998), supporting the notion that this region harbours important tumour suppressor functions. These experiments identified BARD1 as a mediator between pro-apoptotic stress and p53-dependent apoptosis. Thus, in addition to be a coordinator with BRCA1, BARD1 also functions as a tumour suppressor by stabilizing p53 and induces apoptosis, which is BRCA1 independent.

Based on BRCA1-dependent and independent apoptosis functions, a dual mode hypothesis of BARD1 function was raised (Fig. 3) (Irminger-Finger et al., 2001). In the survival mode, BARD1 is involved in DNA repair as heterodimer with BRCA1. In the death mode, BARD1 binds to p53 and induces apoptosis, a function independent of BRCA1. The ratio of BRCA1 and BARD1 may determine the cell fate either going to survive or die (Irminger-Finger and Jefford, 2006; Irminger-Finger et al., 2001).

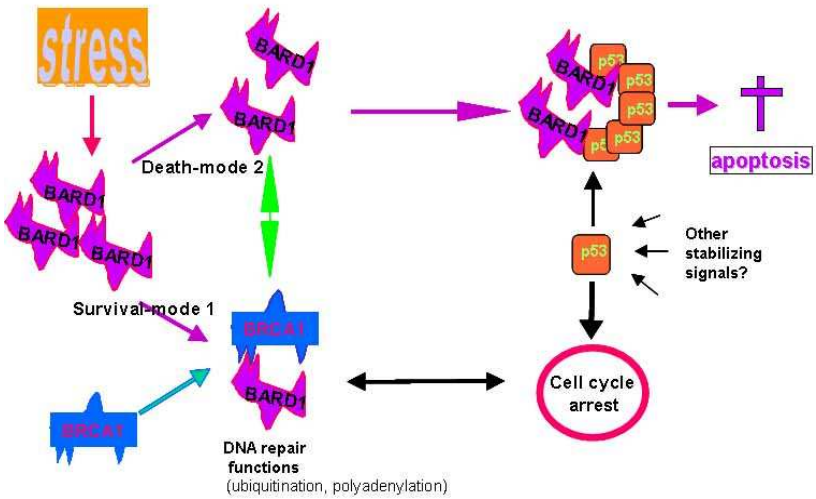


Figure 3. **Presumed dual mode of tumour suppression of BARD1.** Pathway 1: BARD1 is involved in DNA repair as a heterodimer with BRCA1; Pathway 2: BARD1 binds to p53 and induces apoptosis.

Many functions of BARD1 that depend on the BARD1-BRCA1 heterodimer complex have been investigated. The BRCT domains have been reported to confer phosphate binding activity. A potential function of BRCT is to bind to and translocation of phosphorylated substrates of a DNA damage response kinase, such as ATM, (Glover et al., 2004; Manke et al., 2003).

BARD1, independently of BRCA1 interacts with a polyadenylation factor, cleavage stimulation factor (CSTF)-50, which is a protein complex involved in the polyadenylation process of all eukaryotic mRNAs. CstF-50 is required for the endonucleolytic cleavage step of mRNA and it helps to properly identify the site of processing (Takagaki and Manley, 1997; Takagaki et al., 1990). A tumour associated germline mutation in BARD1 (Q564H) results in reduced binding to CSTF-50 and diminished inhibition of polyadenylation (Kleiman and Manley, 2001). DNA damage after hydroxyurea or exposure to UV light may induce BARD1 binding to CSTF1 (Kleiman and Manley, 2001). The addition of BARD1 inhibits CstF-50 polyadenylation, therefore prevents specific RNA processing during DNA damage-induced repair. This provides an explanation for how BARD1 controls cellular proliferation.

Further evidence for BARD1's role in transcription came from the discovery that BARD1 interacts with the NF-κB/Rel transcription factors. NF-κB plays a key role in regulating the immune response to infection. Incorrect regulation of NF-κB has been linked to cancer, inflammatory and autoimmune diseases, septic shock, viral infection and improper immune development. A fragment of BARD1 comprising half of ANK through BRCT domain (residues 464-777) binds in vitro to the ankyrin repeats domain of Bcl-3, which is a member of the IκB family of NF-κB, and modulates the transcriptional activity of the NF-κB complex (Dechend et al., 1999). Indeed, reduced transcription is observed in cells with BARD1 and BRCA1 RING domain mutants (Benezra et al., 2003).

3.3. Novel functions of BARD1 in mitosis

Aberrant or delayed progression of cells through mitosis is an important cause of genetic instability and interferences with cell viability. Previous research has shown that BRCA1 localized to centrosomes during mitosis (Hsu and White, 1998), and BRCA1 defective cells have abnormal numbers of centrosomes (Sankaran et al., 2006). BRCA1 and BARD1 levels increase in mitotic cells, and the proteins are hyperphosphorylated (Choudhury et al., 2005). Therefore, it was speculated that BRCA1-BARD1 might have function in mitosis and in maintaining chromosomal integrity. Recently, a function of BRCA1-BARD1 heterodimer in mitotic spindle assembly, has been demonstrated (Joukov et al., 2006). This study proposes that BRCA1-BARD1 acts through established spindle-assembly factors to control the proper organization of microtubules. It demonstrates that BRCA1-BARD1 ensures fidelity of mitosis and mitotic exit by regulating Ran-dependent (chromatin-driven), spindle assembly. BRCA1-BARD1 attenuates the activity of XRHAMM (Xenopus receptor for hyaluronic-acid-mediated motility) (Groen et al., 2004; Maxwell et al., 2005), thereby permitting the normal concentration of TPX2 (Maxwell et al., 2005; Wittmann et al., 2000) on spindle poles and proper spindle-pole assembly.

Recent data from our lab also demonstrate a role of BARD1 in progression through mitosis/cytokinesis (Jefferd et al., submitted). We observe that BARD1, but not BRCA1, localizes to the spindle during all steps of mitosis and concentrates at the midbridge at cytokinesis, where it interacts with BRCA2, TACC1, and Aurora B. These results provide an explanation for the function of BARD1 in chromosome stability control and tumour suppression.

4. BARD1 mutations and expression in cancer

Cancer is one of the leading causes of death in the world. In fact, it is the second leading cause of death after cardiovascular diseases. Etiology of cancer is not completely defined, but it is well known that genetic factors play very important roles. Cells may become malignant by either over-activation of oncogene, or loss of functions of tumour suppressors.

BRCA1 is one of the most important tumour suppressors accounting for breast and ovarian cancer. Mutations in BRCA1 and BRCA2 are associated with about 50% of familial breast and ovarian cancers. Women harbouring mutations in either BRCA1 or BRCA2 have a 80-90% life-time risk of developing tumours in the breast or in the ovaries (Easton et al., 1993; Easton et al., 1994; Ford et al., 1994; Rahman and Stratton, 1998). About 1,000 mutations, comprising deletions, insertions, missense, and nonsense mutations have been identified in BRCA1.

Since BARD1 is a tumour suppressor, BARD1 mutations should also predispose to cancer. However, BARD1 mutations are less frequent (Fig. 4A). After screening a panel of sporadic breast, ovarian and endometrial cancers, three missense alterations were identified in the BARD1 gene at amino acid positions Q564H, V695L, and S761N (Thai et al., 1998). Loss-of-heterozygosity was accompanied with Q564H and S671N, substantiating BARD1's role as a tumour suppressor. The V695L and S761N mutations were found in somatic breast tissue but not in the germline, whereas the Q564H mutation arose in the germline of a patient with clear cell adenocarcinoma of the ovary (Thai et al., 1998). Five alterations were discovered in an Italian cohort with familial breast and ovarian cancers that was chosen for its absence of BRCA1 and BRCA2 gene alterations in its proband (Ghimenti et al., 2002). These mutations included 3 missense mutations, K312R, C557S, N295S, and an in-frame deletion of 7 amino acid residues, 1139Del21-(PLPECSS). The last alteration is a C1579G transversion with no amino acid change at position A502, which was found in 15 probands, indicative of a novel polymorphism variant (Ghimenti et al., 2002). The mutations C557S and 1139Del21, which

were considered as polymorphisms, were described previously by Thai et al., (1998). However, segregation analysis showed that the C557S mutation might be linked with the tumour with a statistically borderline. Interestingly, many mutations in BRCA1 have been found in the RING finger and disrupt the BRCA1-BARD1 interaction (Wu et al., 1996). On the contrary, BARD1 mutations are mostly around the ANK repeats, the BRCT domains and the region in between these domains (Ghimenti et al., 2002; Ishitobi et al., 2003; Karppinen et al., 2004; Thai et al., 1998).

It is generally accepted that genetic mutations might induce structural changes of the protein product, leading to loss of function due to a presumed decrease of protein stability. Therefore, a decrease of BARD1 expression in tumour cells might be expected. Immunohistochemical analysis of BARD1 expression, on the contrary, found that BARD1 was highly expressed in tumour cells and located in cytoplasm, which is not associated with apoptosis. The aberrant forms of BARD1 expressed in cancers might correspond to aberrant isoforms derived from differential splicing (Wu et al., 2006).

Alternative splicing is a crucial mechanism for generating protein diversity. Different splice variants of a given protein can display different and even antagonistic biological functions. Several genome-wide analyses indicate that more than 50% of human genes present alternative spliced isoforms, suggesting that this mechanism has a major role in the generation of protein diversity (Hu et al., 2001). The connection between splicing and cancer is receiving more attention, and several cancer associated alternations of splicing patterns have been identified, such as Ron, Rac1, Fas, BCL2L1, CD44, MDM2, etc (Pajares et al., 2007; Srebrow and Kornblihtt, 2006).

A BARD1 spliced isoform presenting a deletion of exon 2 through 6 was found in a rat ovarian cancer cell line, which is resistant to apoptosis (Feki et al., 2005) (Fig. 4B). This isoform lacks most of RING domain and the entire span of ankyrin repeats. The same isoform

was later reported in HeLa cells (Tsuzuki et al., 2005). From our lab's previous research, we found that N-terminus was lost in most cases of ovarian cancer, and the extent of up-regulation of BARD1 was correlated with other indicators of poor prognosis, such as tumour type in ovarian cancer and tumour size and stage in breast cancer (Wu et al., 2006).

We therefore hypothesized that tumours might reproduce a developmental pattern of gene expression, which is functional during specific developmental processes. To elucidate BARD1 function in cancer, and to investigate how BARD1 was expressed in cancers and characterize its structure and potential functions in cancer cell growth was therefore the topic of this thesis.

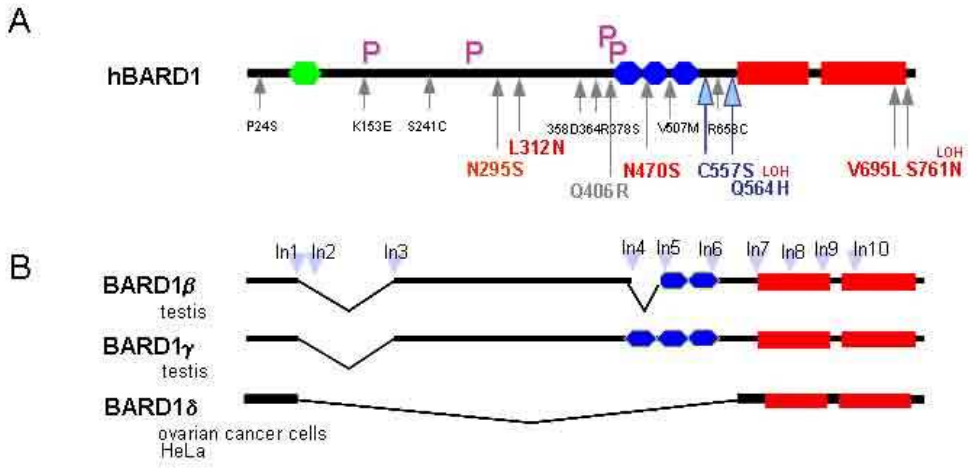


Figure 4. **Mutations and spliced isoforms of BARD1 in cancers.** (A) Mutations in human BARD1. Phopsphorylation sites are indicated (P). Mutations are marked in red, germline mutations in blue, and polymorpisms in black. (B) Splice isoforms of BARD1. BARD1β and γ are expressed in preleptotene spermatocytes (Feki et al., 2004). BARD1δ is expressed in a rat ovarian cancer cell line (Feki et al., 2005) and HeLa cells (Tsuzuki et al., 2005).

5. Cancer like properties of Cytotrophoblasts

To some extend, cancer cell behavior is similar to the proliferation and invasion process of the human cytotrophoblasts (CTBs) (Bischof and Irminger-Finger, 2005). The term "pseudo-malignancy" has been used to describe the properties of the early human placenta, which

refers to the similarities between trophoblast and malignant cancer cells, such as invasiveness, high cellular proliferation rate, lack of cell contact inhibition and immune privilege. CTBs are specialized placental cells that play a pivotal role during the early stage of placental development and embryo implantation. The tumour-like invasion process allows the cells to invade the decidua and myometrium, which is important for gas exchange, nutrition, endocrine function, and immunological support of fetal growth. Many factors are involved in CTB invasion, such as serine proteases, extracellular matrix proteins (Bischof and Irminger-Finger, 2005), matrix metalloproteinases (MMPs) (Westermark et al., 2003), hypoxia (James et al., 2006), and human chorionic gonadotropin (hCG) (Zygmunt et al., 1998). The expression of tumour suppressor genes such as, c-myc, c-erB-2, RB, and BCL-2 has been demonstrated in first trimester placenta (Diebold et al., 1991; Jokhi et al., 1994; Kim et al., 1995; Ohlsson and Pfeifer-Ohlsson, 1986), and these genes may have a role in the control of trophoblast cell population expansion as trophoblast invasion occours.

In this study, we first performed experiments on CTBs of early pregnancy, a model recapulating cancer cell invasion, and found that BARD1 spliced isoforms were expressed in CTBs and might play a role in proliferation and invasion. The second part describes experiments that characterize BARD1 expression pattern in various types of cancer cells and determine their structure and potential function in cancer cell growth.

Materials and methods

1. CTB purification and cell culture

Placental tissues were obtained from patients who voluntarily and legally chose to terminate pregnancy during the first trimester (7–12 weeks of gestation). Informed written consent was obtained from all patients before their inclusion in the study, for which approval was obtained from the local ethic committee.

Cytotrophoblasts were isolated from first trimester placenta as described (Bischof et al., 1995). In brief, fresh tissue specimen were isolated and washed several times in sterile HBSS. Tissue was then enzymatically digested 4 times for 20 minutes at 37°C (0.25% trypsin, 0.25mg/ml Dnase I). Single cells were collected, trypsin coktail was neutralized with FCS (Gibco, Basel, Switzerland), and cells were then resuspended in DMEM (Jin et al., 1997) (Gibco, Basel, Switzerland). This cell suspension was filtered on 100μM filter, laid onto Percoll (GE Healthcare, Uppsala) gradient (70% to 5% Percoll diluted with HBSS) and centrifuged for 25 min at 1200 × g. The 30-45% bands containing cytotrophoblastic cells were collected, washed and suspended in DMEM. Cells were then immunopurified.

Purified CTBs were cultured at normal oxygen level and under hypoxic conditions. The normoxic cells were incubated at 37°C, 20% oxygen, and 5% carbon dioxide atmosphere. Hypoxic condition was achieved using Oxoid Campygen (Oxoid Ltd, Hampshire, UK) in a compact plastic pouche. Oxoid Campygen will absorb oxygen and produce carbon dioxide and then the oxygen and carbon dioxide concentration will around 6% and 10% to 15% in the plastic pouche, respectively. This plastic pouche was incubated at 37°C, 20% oxygen, and 5% carbon dioxide. Cultures were maintained for 72 hours. Media were not changed during incubation period.

2. RNA extraction, reverse transcription and PCR

RNeasy Mini Kit (Qiagen, Switzerland) was used for isolation of total RNA from the collected cultured cells. For reverse transcription, 1 µg of RNA was used in final volume of 20ul, containing reverse transcription buffer, 1µl of oligo dT (500µg/ml), 1 µl of 10mM dNTP's, 2 µl of DTT (0.1M), 4 µl of 5X First standard Buffer and 1 µl of Superscript II (200u/ µl). The reaction was incubated at 65°C 3 minutes followed by 42°C 50 minutes and 70°C 15 minutes. 2 µl of cDNA was used as a template for PCR with different primers for amplification of different regions of BARD1. It was performed with *Taq polymerase* in a final volume of 50ul. Primary denaturation (94°C, 3 min) and final extension (72°C, 10 min) were the same for each PCR. Annealing temperature and extension time were variable according to different primers and length of the expected product (Table 1). Forward and reverse primers for β-hCG were: 5'-TCACTTCACC GTGGTCTCCG-3'; 5'-TGCAGCACGC GGGTCATGGT-3'. 30 cycles were performed with annealing temperature 59°C and 30 seconds for extension time. Primers for Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were as described before (Irminger-Finger et al., 2001; Wu et al., 2006). PCR product (15 µl) was used for analysis in 1% of agarose/TAE gel with EtBr and visualized under UV light.

Table 1. Primers and conditions for PCR

Forward primer		Reverse primer		PCR product (bp)	Anealing Tem (°C)	Extension (sec)
sequence	Position (bp) (exon)	sequence	Position (bp) (exon)			
5'GAGGAGCCTTTCATCCGAAG3'	-28 (Ex 1)	5'TTTTGATACCCGGTGGTGTT3'	1481 (Ex 6)	1508	56	100
		5' CAGCTGTCAAGAGGAAGCAAC3'	2333 (Ex11)	2361	56	140
5' GTGACTGCATTGGAACTGGA3'	228 (Ex 3)	5' CAGCTGTCAAGAGGAAGCAAC3'	2333 (Ex11)	2105	55	130
5'AGCAAGTGGCTCCTTGACAG3'	783 (Ex 4)	5' CAGCTGTCAAGAGGAAGCAAC3'	2333 (Ex11)	1550	56	100
5'GAGGAGAGACTTTGCTCC3'	1280 (Ex4)	5' CAGCTGTCAAGAGGAAGCAAC3'	2333 (Ex11)	1053	54	80
5'GCTGGATGGACACCATTG3'	1378 (Ex4)	5' CAGCTGTCAAGAGGAAGCAAC3'	2333 (Ex11)	955	54	70
5'CTCCAGCATAAGGCATTGGT3'	1441 (Ex 6)	5' CAGCTGTCAAGAGGAAGCAAC3'	2333 (Ex11)	892	56	60

3. Cell culture and hCG treatment

Choriocarcinoma cell line JEG-3, and HeLa cells were cultured in collagen-coated tissue culture flasks (Falcon, Becton Dickinson, San José, CA) in low glucose DMEM supplemented with 10% fetal calf serum (FCS), penicillin (110 µg/ml) and streptomycin (110 µg/ml). Cells were treated with HCG (Gonadotropinum Chorionicum 1500) 9 IU/ml and incubated for 48 hours. Cells were trypsinized and collected for RT-PCR and Western blots.

4. Cell culture and ELISA assays

CTB, MCF-7 or MDA-MB231 cells were cultured for two days with or without doxorubicin (10µg/ ml medium) or phorbol 12-myristate 13-acetate (PMA), then cells were harvested and supernatants were collected for detecting BARD1 expression by ELISA.

Goat anti-human BARD1-N19 (sc-7372; Santa Cruz, CA) (500 µl), was diluted 1:1 with bicarbonate buffer (0.1 M, pH 8.4) and dialysed against this buffer for 48 hours at 4 °C. Activated biotin, at a concentration of 10 mg/ml in DMSO, was added (110 µl) and incubated for 2 hours at room temperature. The preparation was dialysed against PBS containing 0.02 % NaN3, and stored at 4°C.

Plates of 96 wells were coated overnight at 4°C with 100 µl of goat anti-human BARD1-C20 (sc-7372; Santa Cruz, CA) (30 µg/ml) in Na-carbonate buffer (50 mM, pH 9.6). Unbound sites were blocked for 2 hours at room temperature with 250 µl of 10 % Blotto in PBS containing 0.02 % NaN3. Plates were then washed twice with PBS containing 0.1 % Tween 20 (PBST, 250 µl/ well) and once with PBST plus 10 % Blotto (PBSTB).

Samples and standards, diluted in PBS containing 10 % Blotto (PBSB), were applied in duplicates (100 µl/ well) and incubated overnight at room temperature. After incubation, the plates were washed and incubated with biotinylated N-19 (100 µl/well) for 2 hours at room

temperature on a rotating platform. Plates were then washed 3 times with PBST, and once with PBSTB and re-incubated for 30 min at 20°C with avidine-peroxydase (1/4000 in PBSTB, 100 µl/ well).

After washing (4 times) with PBST, the plates were incubated in the dark for 10 minutes with OPD and H_2O_2 30% (10 mg and 10 µl/25 ml) in citrate-phosphate buffer (0.05 M, pH 5.0, 200 µl/well). The reaction was stopped by the addition of sulphuric acid (3M, 50 µl/ well) and the absorbance measured at 492 nm in an ELISA plate reader (Labsystem Multiscan, BioConcept, Allschwill, Switzerland).

5. Western blot

Cells were directly lysed in Ripa buffer and 40 µg of protein were loaded per lane on 10% SDS-PAGE and blotted onto PVDF filters. Membranes were blocked with 5% milk powder in 0.05% PBS-Tween. Synthetic peptides corresponding to amino acid residues 83 to 95 and 145 to 160 of BARD1 were used to generate polyclonal antibodies designated PVC and WFS in rabbits. Antibody JH3 was rabbit polyclonal antibody recognizes amino acid residues 526 to 542. A designated antibody H300 (sc-7372; Santa Cruz, CA) was used for detecting N-terminus, and C20 (sc-7372; Santa Cruz, CA) was used to detected C terminus. Anti-Bard1 H300, PVC, WFS, JH3, and C20 were used as first antibodies, incubated at 4 °C overnight. Secondary anti-rabbit or anti-Goat peroxidase-coupled antibodies were applied in a 1:10,000 dilution. Signal detection was performed with the enhanced chemiluminescence kit (Amersham, Arlington Heights, IL).

6. *CTBs and TAC2 cells co-culture*

CTBs were purified as described above. TAC-2 cells were cultured on collagen-coated Petri dishes (Irminger-Finger et al., 1998). Cells were co-cultured or cultured individually with

standard medium. CTBs were transfected with antisense BARD1 oligos by standard transfection procedures and supernatants of treated and untreated cells were collected after 48 hours. CTB supernatants were applied to TAC-2 cultures from untreated or anti-sense oligos transfected cells and cells were cultured for 24 hours. Cell adhesion and survival was monitored by microscope and adherent cells were counted and compared. Sequence of antisense and sense oligos was 5'AGCTTTTCCA AAAAGTGTAT GCTTGGGATT CTCTCTCTTG GAAGAGAATC CCAAGCATAC ACGGG3' and the equivalent sense sequence.

7. *Cancer cell lines*

Cancer cell lines were generously provided by Dr. R. Zeillinger, Universitätsspital Wien.

Brest cancer cell lines (B1-B26) were: MCF-7, MM231, T47D, Hs578T, SKBR3, MM435s, ZR-75-1, BT549, MM453, BT474, PA1, A2780ADR, BT20, HBL100, HMEC, MCF12A, MCF10A, MCF7/6, MCF12F, MM134VI, MM157, MM175VII, MM330, MM468, UCAA812, MM361.

Cervical cancer cell lines (C1-C9) were: HeLa, SW756, GH354, Ca Ski, C-4 I, C-33 A, HT-3, ME-180, SiHa.

Endometrial cancer cell lines (E1-E9) were: KLE, RL95-2, AN3 CA, HEC-1-B, Ishikawa, Colo 684, HEC-50, EN, EJ.

Ovarian cancer cell lines (O1-O32) were: A2780, Caov-3, ES-2, NIH:OVCAR-3, SK-OV-3, TOV-21G, TOV-112D, OV-90, OV-MZ-1a, OV-MZ-1c, OV-MZ-2, OV-MZ-2a, OV-MZ-5, OV-MZ-6, OV-MZ-8, OV-MZ-9, OV-MZ-10, OV-MZ-12, OV-MZ-12b, OV-MZ-17b, OV-MZ-18, OV-MZ-20, OV-MZ-21, OV-MZ-22, OV-MZ-26, OV-MZ-27, OV-MZ-30, OV-MZ-32, OV-MZ-33, OV-MZ-35, OV-MZ-37, OV-MZ-38.

8. Mapping of mRNA 5'end

GeneRacerTM Kit (invitrogen) was used to amplify 5' cDNA end from RNA of ovarian cancer patient and HeLa cells. Total of 4.5µg of RNA was used. Then, the total RNA was treated with calf intestinal phosphatise (CIP) to dephosphorylate non-mRNA or truncated mRNA. Remove the mRNA 5' cap structure and ligate the RNA oligos to decapped mRNA. Then, reverse transcription reaction was performed to get the cDNA. In order to amplify the 5' cDNA end, first PCR was performed with 5' race primer (5'-CGACTGGAGC ACGAGGACACTGA-3') and reverse primer in exon11 (5'-GTTGCCAAAGCTGTTTG-3'). 5' nested PCR was performed with 5' nested primer (5'-GGACACTGAC ATGGACTGAA GGAGTA-3') and reverse primer in exon6 (5'-TTTTGATACC CGGTGGTGTT-3'). All these procedures were performed according to the manufacturer's instructions. The PCR bands of 5' nested PCR were loaded on 1% low melting gel, cut, and purified with the QIAEX II kit (Qiagen, Hombrechtikon, Switzerland) followed by sequencing with 5' nested primer and reverse primer.

9. Purification of DNA and sequence with pGEMT cloning

The QIAEX II kit (Qiagen, Hombrechtikon, Switzerland) was used for DNA purification of RT-PCR. Purified DNA was cloned into pGEMT Easy vector (Promega, Madison, WI). Ligation and transformation were performed according to the manufacturer's instructions. The insert/vector ratio was 3:1. Two microliters of the ligation reaction was mixed with 50 µl of JM109 High Efficiency Competent Cells in LB were plated onto L-broth/ampicillin/PTG/X-Gal plates and incubated at 37°C overnight. Recombinant clones could be identified by colour screening on indicator plates. We chose 5 white colonies in each plate and incubated them in 3 ml of LB with ampicillin at 37°C overnight. Recombinant plasmid DNA was isolated using the Miniprep kit (Sigma, St. Louis, MO), followed by sequencing with primers T7 and SP6.

10. Immunohistochemistry

Formalin-fixed and paraffin-embedded 5 μm tissues sections and micro tissue arrays were deparaffinized with xylene for 48 hours, and rehydrated through descending alcohol concentration (100% alcohol, 95% alcohol, 70% alcohol, H2O). The sections were boiled 5 minutes in microwave for antigen retrieval, and then blocked with the endogenous peroxidase. Slides were incubated overnight or for 24 hours at 4°C in a humidifying chamber with the first antibody after BSA (bovine serum albumin) blocking the nonspecfic proteins. The primary antibodies used for BARD1 detection were N19 (sc-7373, Santa Cruz Biotechnology) (1 : 25 diluted), PVC (1 : 100 diluted), WFS (1 : 100 diluted), described previously (Irminger-Finger et al., 1998) and C20 (sc-7372, Santa Cruz, CA) (1 : 20 diluted), which recognize N-terminal, epitope in exon 3, exon 4, and C-terminal epitopes of BARD1, respectively. BRCA1 antibody was D16 (Santa Cruz Biotechnology), recognized an N-terminal epitope. Secondary antibodies (goat anti-rabbit or rabbit anti-goat) conjugated with horse radish peroxidase (HRP) were applied in 1:100 dilutions at room temperature for 1 hour. Then diaminobenzidine (DAB) staining was permitted for 15 minutes at room temperature. Slides were counter stained with hematoxylin before dehydration and mounting.

To quantify BARD1 expression, staining was scored by intensity and percentage of the stained cells. The value of the staining intensity and positive cell percentage times together gets the final staining score. Statistical significance of comparison was determined by applying student t test.

11. Clinical data

The pathological diagnosis were made by experienced pathologists and staged according to the WHO and AJCC classification. There are 106 cases of ovarian cancer from women of 32-

87 years old, were analyzed, comprising of 60 cases of serous carcinoma, 24 cases of endometriod carcinoma, 16 cases of mucinous carcinoma, and 6 cases of clear cell carcinoma. According to TNM staging system, there were 38 cases in T1; 15 cases in T2; 53 cases in T3; 36 cases in N0, and 63 cases in N1 stage. There were 25, 26, and 55 cases of pathologic grade 1 to 3, respectively.

A total of 145 of colon cancer cases were studied in this analysis. There were 3 cases in T1, 32 cases in T2, 68 cases in T3, 42 cases in T4. There are 66 cases with lymph node metastasis, 70 cases without lymph node metastasis.

12. Promoter methylation

A total of 50 primary epithelial ovarian cancer tissues and 34 ovarian cancer cell lines were used as described previously (Pils et al., 2005). Genomic DNA, 500 ng of primary tissues and 1 µg of cell lines, was treated with bisulfite as described previously (Horak et al., 2005). PCR primers specific for the methylated and unmethylated CpG islands after bisulfite treatment were designed using MethPrimer (http://www.urogene.org/methprimer/) and evaluated on artificially methylated genomic DNA (Sss I Methylase). 30 ng bisulfite treated DNA was used as template for MSP (primers and PCR conditions will be provided upon request). PCR products were analyzed on 3% agarose gels and visualized with Ethidium bromide. Artificially methylated DNA (Sss I Methylase) served as positive control.

Results

1. Structure of BARD1 isoforms

To unravel the expression pattern of BARD1, we first determined the structure of BARD1 isoforms in human normal fibroblasts and in HeLa cells by RT-PCR. BARD1 was highly expressed in normal fibroblasts, and corresponded to the full length (FL) BARD1, when primers for amplify of the entire coding region were used. In HeLa cells, however, spliced isoforms of BARD1 were expressed together with FL BARD1 (Fig. 5A). We cloned and sequenced these isoforms and determine their structure, exon composition, and calculated molecular weight (MW) (Fig. 5B). FL BARD1 translates into a protein of 777 amino acids or a calculated MW of 87 kD. Isoform α has a deletion of exon 2 and produces a 85 kD protein of 758 amino acids. Isoform β, derived from deletion of exon 2 and 3, translates into a protein of 680 amino acids or 75 kD, but would use a translation start in an alternative reading frame of exon 1 (Fig. 5C). Deletion of exon 4 in isoform γ disrupts the open reading frame. However, isoform φ and δ, missing exons 2 to 6 or 3 to 6, could produce a 37 or 35 kD protein of 326 or 307 amino acids. Isoform δ was reported previously in HeLa (Tsuzuki et al., 2005) and ovarian cancer cells (Feki et al., 2005). Isoform ε is lacking exons 4 to 9 with a predicted MW of 30 kD, composed of 264 amino acids, and isoform η is composed of exons 1, 10, and 11, which is not in frame but initiation of translation could occur in an alternative reading frame and translate into a 19 kD protein of 167 amino acids (Fig. 5C). All these spliced isoforms might either loose RING domain or ANK or/and part of first BRCT domain, which are the important functional regions for BARD1 as a tumour suppressor. Therefore, we were interested in investigating expression of these isoforms further to elucidate their functional role in cancer development and progression.

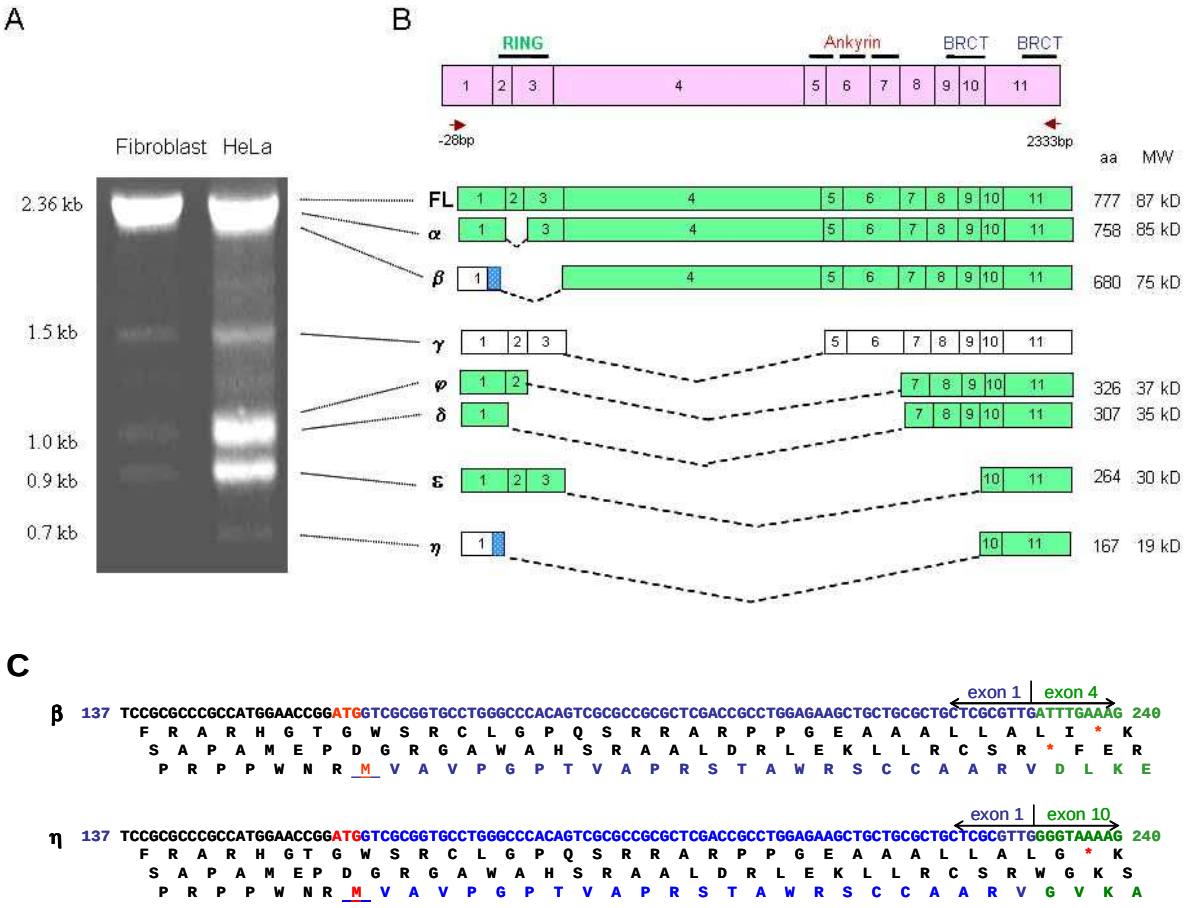


Figure 5. **Structure of BARD1 isoforms.** (A) Amplification of FL BARD1 in normal skin fibroblasts and HeLa cells by RT-PCR. (B) Diagram of BARD1 exons and structural domains compared to exon structure of FL BARD1 and isoforms α, β, γ, φ, δ, ε, and η. Approximate locations of structural domains are indicated as RING, Ankyring, and BRCT above BARD1 molecule structure. Small arrows mark positions of forward and reverse primers used for RT-PCR. Open reading frame corresponding to known BARD1 sequence is presented in green (filled) boxes, alternative reading frame (ORF) is indicated in blue (spotted) boxes, and out of frame, non-coding regions are unmarked. Amino acids and calculated molecular weight are indicated. (C) Sequences of splice junctions of isoforms β and η are presented. Known BARD1 ORF is marked green, alternative ORF blue. Possible translation initiation methionines of isoforms β and η are labelled red within alternative ORF of exon 1.

2. Expression of BARD1 in cytotrophoblasts of early pregnancy

Since the human cytotrophoblast has invasive properties reminiscent of cancer cells, we used CTBs of early pregnancy as a model of cancer to investigate BARD1 expression and functions.

2.1. Temporal expression of BARD1 in CTBs of early pregnancy

In a first approach, RT-PCR was performed by amplifying FL BARD1 to determine its expression in purified CTBs from different weeks of early pregnancy (Fig. 6). When investigating expression levels in CTBs from 7 to 12 weeks of pregnancy, we found that BARD1 expression level varied. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was tested in each sample and its expression remained unchanged. Interestingly, we found that together with FL BARD1, spliced isoforms were also detected in CTBs, such as isoform β, δ, ε and η.

Thus, we found that FL BARD1 and spliced isoforms are expressed in CTBs in early pregnancy, and expression reaches a peak level at 9 weeks of pregnancy and decreases afterwards. This finding suggested a temporal control of transcriptional regulation of BARD1 expression during the first trimester of placental development.

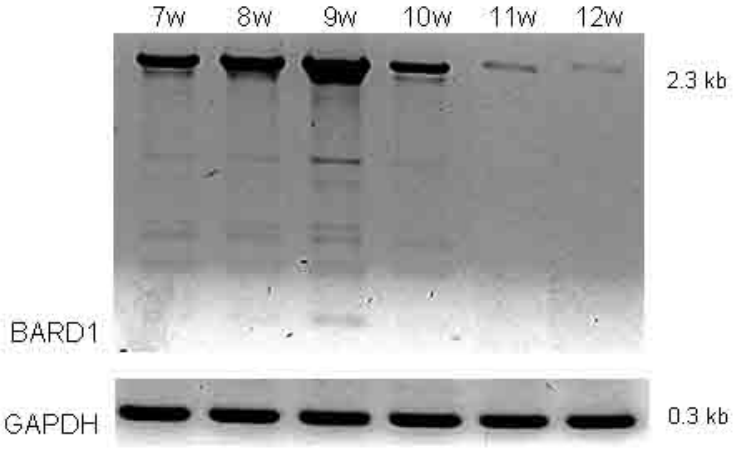


Figure 6. **BARD1 expression analysis in CTBs at different weeks of pregnancy by RT-PCR.** BARD1 cDNA coding region was amplified and compared to GAPDH expression. At least 3 different samples were tested for each week of pregnancy, and representative samples are presented.

2.2. HCG regulates BARD1 expression in CTBs

A major modulator of placenta development is the human chorionic gonadotropin (hCG), a hormone associated with the initiation and maintenance of pregnancy, by inducing the invasive behaviour of CTBs (Oktay et al., 1994; Zygmunt et al., 1998). HCG levels change during the early weeks of pregnancy and reach a peak level at 8 to 10 weeks, then decline for the remainder of the pregnancy.

Since BARD1 expression also showed a peak at 9 weeks of pregnancy, it was interesting to investigate the relationship between BARD1 and hCG expression in isolated CTBs. We performed RT-PCR to determine the expression of the inducible subunit of hCG, β-hCG (Miller-Lindholm et al., 1997) and compared it to BARD1 expression. Indeed, β-hCG levels paralleled the expression of BARD1 in our samples; they were high at 7 to 9 weeks and decreased from 9 to 12 weeks (Fig. 7A). This finding raised the question whether BARD1 transcription might be induced by hCG.

To test this hypothesis, we investigated whether BARD1 expression was under hCG control in *in vitro* cell cultures. We used the choriocarcinoma cell line JEG-3, known to express hCG and its receptor, and HeLa cells, which do not express hCG or its receptor, as control cells. RT-PCR of the inducible subunit β-hCG was performed to confirm that JEG-3 cells expressed β-hCG but HeLa cells did not (Fig. 7B). Cells were cultured for 48 hours with or without addition of purified hCG. RT-PCR was performed to determine BARD1 expression with primers amplifying the overall coding region of FL BARD1. We observed an increase of FL BARD1 mRNA expression after hCG treatment in JEG-3 cells. Interestingly we also found that smaller, deletion bearing isoforms of BARD1 were upregulated with hCG treatment. As expected, BARD1 expression did not change in HeLa cells treated with hCG (Fig. 7C). RT-PCR of GAPDH showed identical expression levels for all samples. To confirm this finding, we performed Western blots analysis, using BARD1 antibody C20, directed against an epitope at the BARD1 C-terminus, on protein extracts from these cultured cells, and

compared BARD1 protein expression (Fig. 7D). Based on the mRNA structure we deduced the molecular weight of the isoforms identified by RT-PCR (Fig. 5)), i.e. FL BARD1 as 97 kD protein, and isoforms δ, ε, and η with their respective molecular weight. Expression of these isoforms was increased in JEG cells after hCG treatment. However, in HeLa cells, which do not react to hCG, no increase after hCG treatment was observed, which is consistent with the result of RT-PCR. These results indicated that hCG was a positive regulator of BARD1 expression.

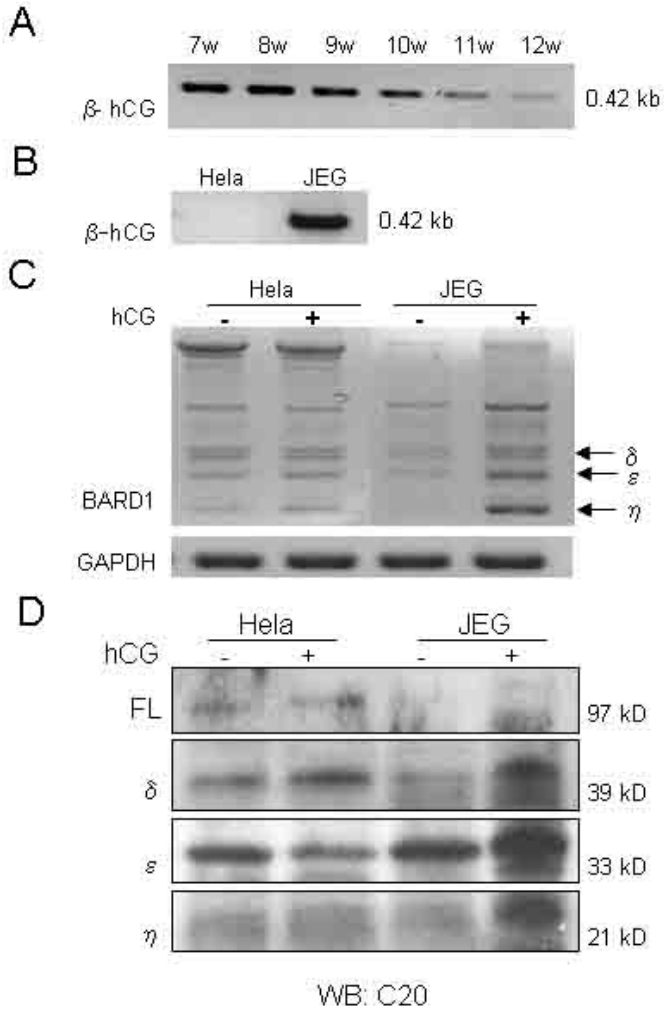


Figure 7. **HCG regulates BARD1 expression.** (A) Test for β-hCG expression at different weeks of pregnancy by RT-PCR. Same samples were used as presented in Figure 6. (B) β-hCG expression was tested in HeLa and JEG-3 choriocarcinoma cells. (C) RT-PCR analysis of BARD1 expression in HeLa and JEG-3 cells cultured with (+) and without (-) hCG treatment. Control expression of GAPDH is shown underneath. (D) Western blot analysis of samples shown in (C), using BARD1 antibody C20. Proteins of 97, 39, 33, and 21 kDa, were observed, which correspond to the predicted molecular weights of FL BARD1, δ, ε, η.

2.3. Hypoxia induces BARD1 expression in CTBs of early pregnancy

While hCG might be a regulator of CTB invasion, another local modulator is the oxygen supply. It is well established, that during the first trimester, the placenta develops in an

environment of physiological hypoxia (Rodesch et al., 1992); placental oxygen is low at 9 weeks, and after 10 to 12 weeks of gestation it increases (Jauniaux et al., 2000). Therefore, we wondered whether changing expression levels of BARD1 in purified CTBs during different weeks of pregnancy could reflect the changing oxygen levels. Thus we tested BARD1 expression in CTBs under different levels of oxygen. Purified CTBs from different weeks of pregnancy in first trimester pregnancy were cultured under normoxic and hypoxic conditions and RT-PCR was performed to analyze BARD1 expression (Fig. 8). We found that FL BARD1 and isoforms δ, ε, and η expression was increased in hypoxia as compared to normoxia at different weeks of pregnancy. In CTBs of 10 weeks of pregnancy BARD1η is especially upregulated by hypoxic conditions. Since hypoxia can upregulate BARD1 expression in CTBs, and hypoxia is also known to increase the invasion capacity of CTBs, we hypothesized that BARD1 and its isoforms might be involved in regulating the invasive ability of CTBs.

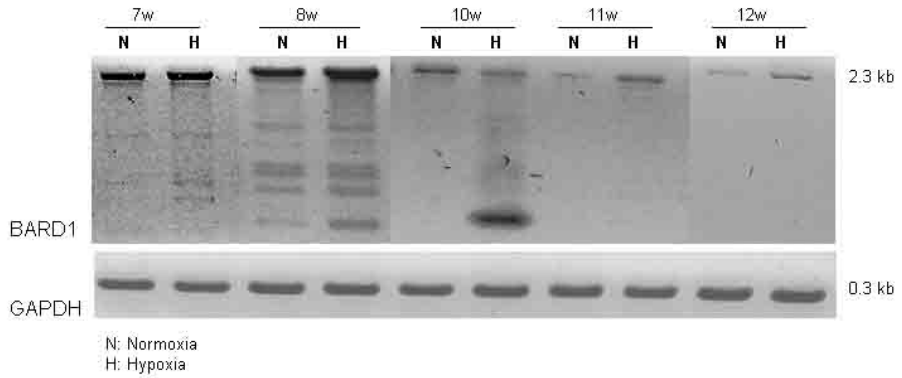


Figure 8. **BARD1 expression in CTBs at different weeks of pregnancy cultured under normoxic and hypoxic conditions was monitored by RT-PCR.** Control expression of GAPDH was assayed in the same samples. Note that BARD1η is upregulated under conditions of hypoxia in CTBs of 10 weeks of pregnancy.

2.4. Locally regulated expression of BARD1 in first trimester placenta

In the first trimester of human pregnancy, the placenta has a villous structure. It contains fetal blood vessels in a core of mesenchymal connective tissue, surrounded by an inner layer of

mononuclear villous cytotrophoblasts. Villous CTBs can afterwards differentiate to overlain multinucleated syncytiotrophoblast or extravillous cytotrophoblast, which break through the syncytiotrophoblast and are highly invasive (Aplin, 1991). To further define BARD1 expression in human CTBs of early pregnancy, we applied immunohistochemical staining to study its local expression. Different antibodies directed against epitopes at the N-terminus (N19), exon 3 (PVC), exon 4 (WFS), and C-terminus (C-20) of BARD1 were used, and from their particular staining pattern we deduced which isoform was expressed in a particular region (Table 2). We found that N19 (Fig. 9A), and C20 showed strong staining in CTBs at different weeks of pregnancy, while PVC and WFS did not always result in positive staining. In villous CTBs at 5 weeks, both PVC and WFS were negative, from what one can deduce expression of isoform δ; the same isoform was also found in villous CTBs at 12 weeks (Fig. 9B). In extra-villous CTBs, which are more invasive, we found that BARD1 was expressed in the cytoplasm and perinuclear region. N19 and C20 were both positive at 5 weeks, while PVC was negative and WFS was positive, an expression pattern difficult to attribute to a specific isoform (Fig. 9C). At 12 weeks, PVC was positive and WFS was negative, which can be interpreted as expression of isoform ε (Fig. 9C) (Table 2).

Table 2. Immunohistochemistry of BARD1 in human placenta

	Cell type	Antibodies				isoforms
		N19	PVC	WFS	C20	
5w	Villous CTB	++	-	-	++	δ
	Extravillous CTB	++	-	+	++	Unknown
12w	Villous CTB	++	-	-	++	δ
	Extravillous CTB	++	+	-	++	ε
12w	Proliferative CTB	++	-	-	++	δ
	Invasive CTB	++	+	-	++	ε

We further analyzed the extravillous cell columns comprising proliferative CTBs and invasive CTBs (Fig. 10). As illustrated in Figure 10, invasive CTBs transit a zone of hypoxia, which is

responsible for inducing CTB proliferation (Fig. 10, P-CTBs) (Bischof and Irminger-Finger, 2005). Interestingly, we found that in this zone of proliferate CTBs isoform δ was expressed, and isoform ε was expressed in the zone of invasive CTBs (Fig. 10, Inv-CTBs). These staining patterns clearly showed that BARD1 isoforms were spatially controlled by hypoxia. In parallel, we performed BRCA1 immunohistochemical staining and found that BRCA1 was barely expressed in CTBs (Fig. 9 & 10). These results demonstrate that in different regions of the placenta-endometrium interface, different BARD1 isoforms are expressed to which different functions can be attributed, i.e. isoform δ expressed in proliferate CTBs and isoform ε in invasive CTBs.

Since BARD1 was expressed in invasive CTBs, and its expression is upregulated by hypoxia and hCG, we wondered whether *in vivo* upregulation of hCG was also associated with an upregulation of BARD1. To address this question, we performed immunohistochemistry on sections of choriocarcinoma or hydatidiform moles which are associated with hundred folds upregulated serum levels of hCG. In all cases we found high levels of BARD1 expression, confirming that BARD1 is upregulated in the presence of hCG *in vivo*. BARD1 showed a distinct pattern of expression with different antibodies in choriocarcinoma cells (Fig. 11). Strong N-19 and C20 staining was found perinuclear and in the cytoplasm, but PVC and WFS staining was weak and localized both to the cytoplasm and nuclei, which could be interpreted as moderate FL BARD1 expression and elevated expression of isoforms missing epitopes for exon 3 (PVC) and exon 4 (WFS).

Together these data demonstrate that BARD1 is highly expressed in CTBs of early pregnancy and associated with invasion, and that hypoxia and hCG are possible inducers of BARD1 expression. Furthermore, these data suggest that different isoforms of BARD1 might be expressed in a timely and spatially regulated manner and play a role in CTBs proliferation and invasion.

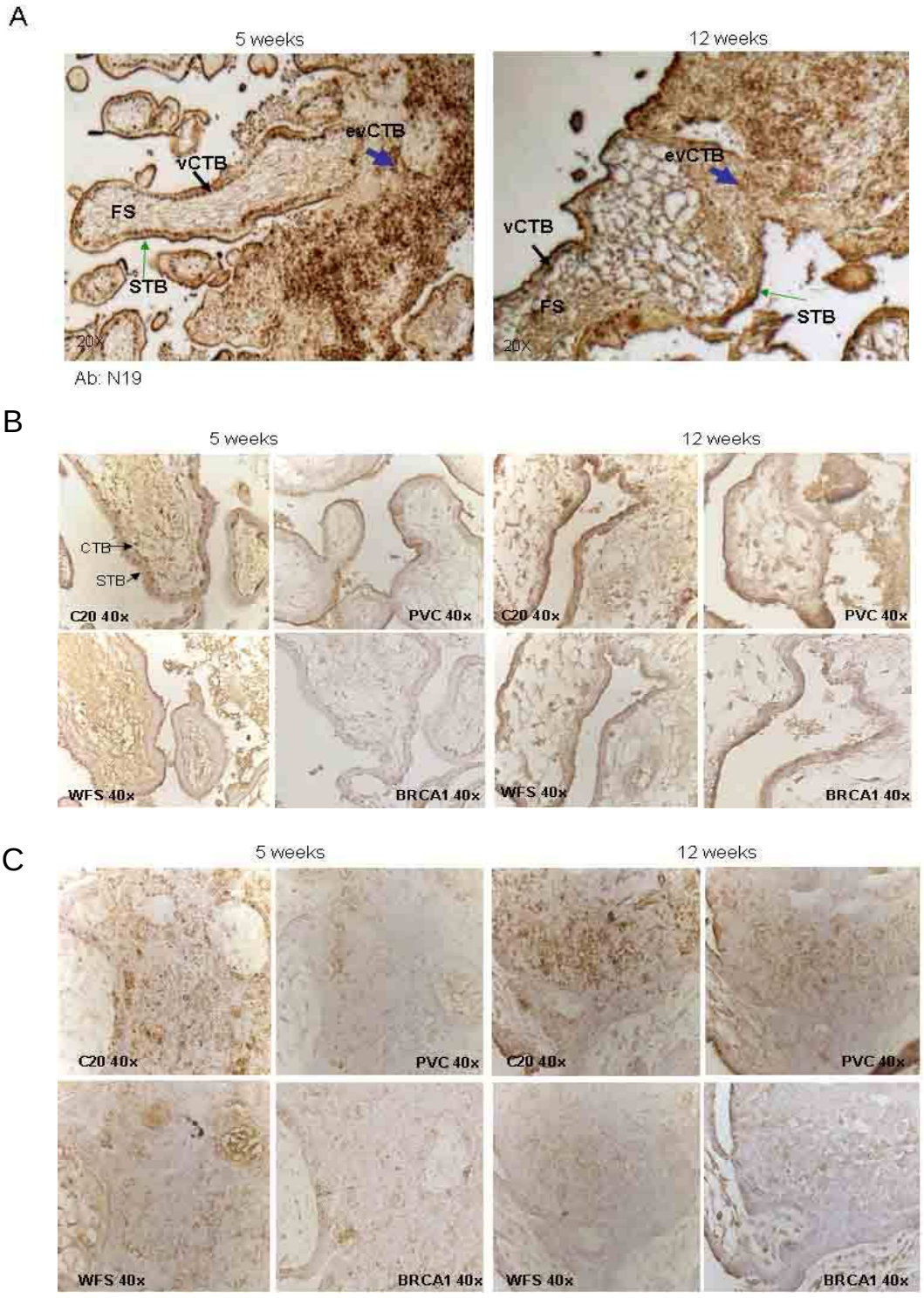


Figure 9. **Comparison of BARD1 expression in human placenta by immunohistochemistry at 5 and 12 weeks of pregnancy.** (A) N19 (directed against BARD1 N-terminus) staining is shown in villous (vCTBs) and extravillous CTBs (evCTBs) at 5 and 12 weeks of pregnancy. Thin black arrows indicate vCTBs, thin green arrows indicate syncytiotrophoblasts (STBs), and thick blue arrows indicate evCTBs; fœtal stroma (FS). (B) Comparison of different BARD1 epitopes, namely C-terminus (C20), exon 3 (PVC), exon 4 (WFS), and BRCA1 expression in vCTBs at 5 and 12 weeks of pregnancy. (C) Comparison of different BARD1 epitopes and BRCA1 expression in evCTBs at 5 and 12 weeks of pregnancy.

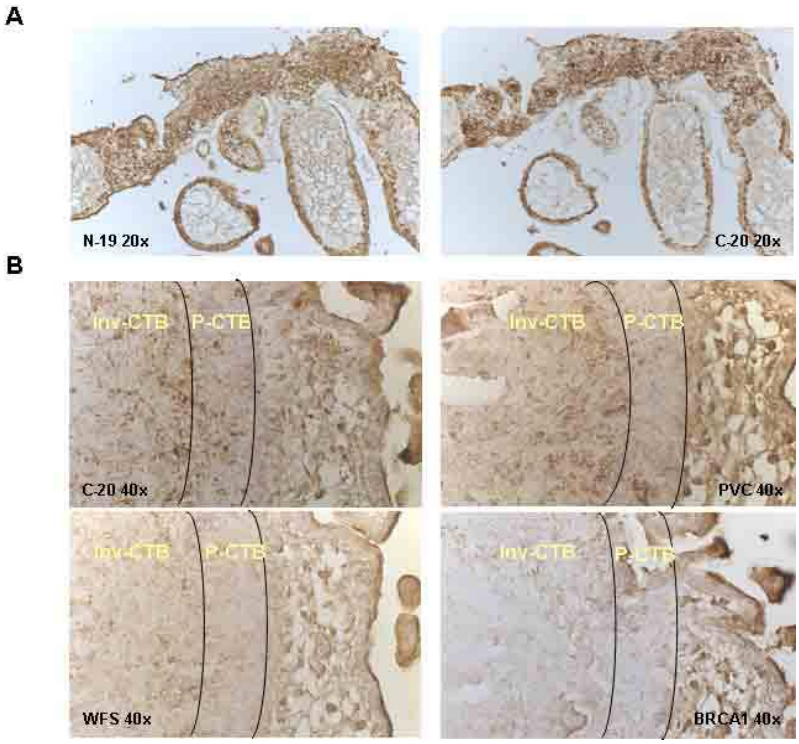


Figure 10. **Regionally controlled BARD1 expression in extravillous CTBs.** (A) Epitopes detected by N19 and C20 are highly expressed in extravillous CTBs. (B) Comparison of expression of different BARD1 epitopes, using C20 (C-terminus), PVC (exon3), WFS (exon 4), and BRCA1, in proliferative CTBs and invasive CTBs. Black lines mark region of proliferative cells (P-CTB) growing in hypoxic conditions, in more profound regions invasive CTBs (inv-CTBs) are found. The expression of particular BARD1 isoforms in distinct regions is concluded from this specific expression pattern, as summarized in Table 2.

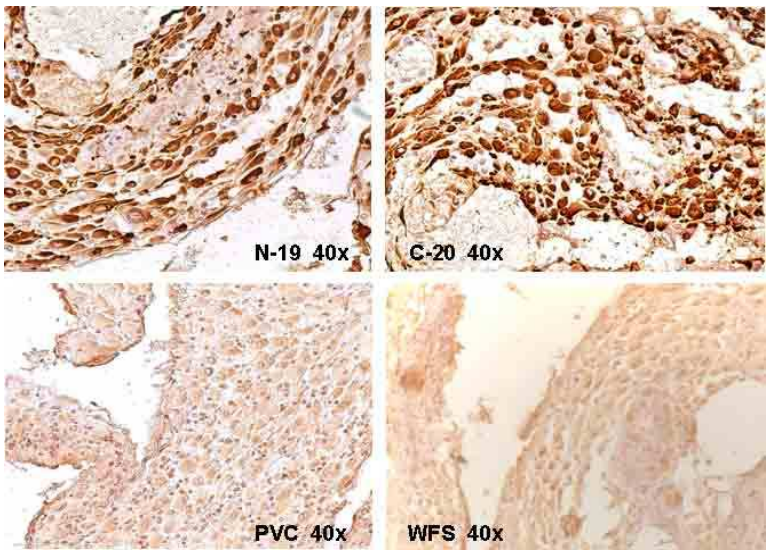


Figure 11. **BARD1 is highly expressed in choriocarcinoma.** Immunohistochemistry with different antibodies demonstrates that N-terminal and C-terminal epitopes are highly expressed as compared to epitopes detected by PVC (exon 3) and WFS (exon 4).

2.5. Secreted BARD1 isoforms

Trophoblast cells are known to secrete a number of proteins important for extracellular matrix remodelling, such as MMPs and hCG, which are important for successful implantation and are suspected to act in one pathway. Since BARD1 was localized to the cytoplasm in invasive CTBs, it was interesting to test whether BARD1 was also secreted by CTBs. We measured BARD1 concentrations in supernatants of cultured cells. Purified CTBs, MCF-7, MDA-MBA231 cells were cultured under normal condition and were treated with doxorubicin or phorbol 12-myristate 13-acetate (PMA) to increase BARD1 expression levels (Irminger-Finger et al., 2001). ELISA assays were performed with cell lysates and cell supernatants to monitor the level of BARD1 protein. In cell lysates of CTBs, BARD1 expression was 20 fold higher than in the other two cell types. Doxorubicin and PMA could induce even higher expression levels of BARD1 in CTBs. BARD1 protein was also found in the supernatant of CTBs, and it also increased after doxorubicin or PMA treatment. Only very low levels of BARD1 protein were found in the supernatants of MCF-7 and MDA-MBA231 cells (Fig. 12A).

To confirm these findings, cell extracts and supernatants of CTBs were probed on Western blots with different antibodies (Fig. 12B). In the supernatant of CTBs anti-BARD1 antibody PVC, but not WFS, detected a protein which, based on size and presence or absence of epitopes, corresponded to BARD1ε. Interestingly, we identified BARD1ε as isoform which is specifically expressed in invasive CTBs (Table 2). Another protein of 82 kD, detected with BARD1 antibody WFS, but not PVC, might represent BARD1β, as was demonstrated with specific repression by exon-specific BARD1 siRNAs (Jefford et al., submitted). Antibody C20 detected both BARD1β and ε and a few proteins corresponding to unidentified protein isoforms or degradation products of BARD1. Thus, at least two isoforms of BARD1 were

specifically detected in the supernatant of cultured CTBs, namely BARD1 isoforms β and ε, and it is ε which is expressed in invasive CTBs.

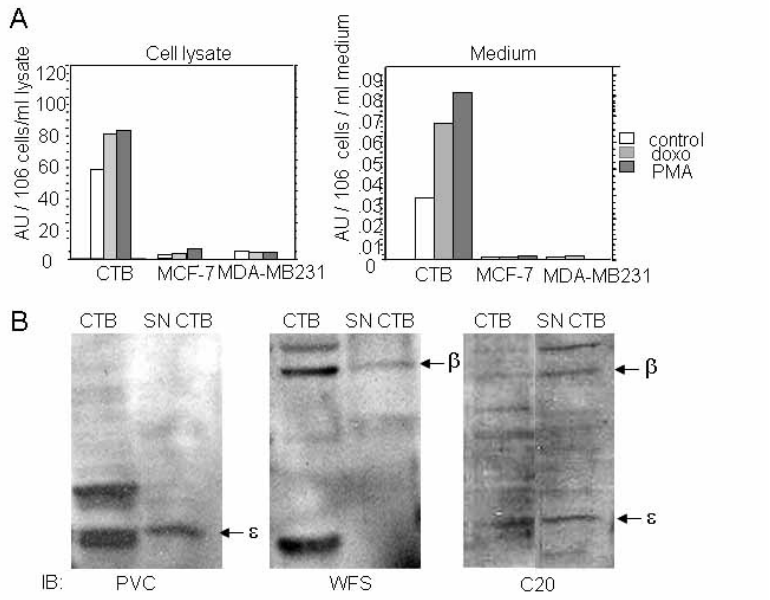


Figure 12. **Detection of secreted BARD1 isoforms.** (A) Detection of BARD1 protein by ELISA, using N19 and C20 antibodies, in cell lysates and supernatants (medium) of CTB and breast cancer cell lines MCF-7 and MDA-MB231. Untreated and doxorubicin (doxo) or phorbol 12-myristate 13-acetate (PMA) treated cells were used. (B) Western blot analysis of BARD1 expression in cell lysates of CTBs and cell culture supernatants (SNCTB). Proteins compatible with the molecular weight of BARD1ε, and BARD1β are detected with PVC (exon 3) and WFS (exon 4) antibodies, respectively. Antibody C20 recognizes both isoforms.

2.6. A role for secreted BARD1 in cell adhesion

Since BARD1 splice variants were found in the supernatant of CTBs, and since the expression profile of BARD1 correlated with and was influenced by factors known to increase the invasiveness of CTBs, we hypothesized that the secreted isoforms of BARD1 might be involved in mechanisms that favour invasion. To test this hypothesis, we co-cultured CTBs with epithelial cells TAC2. TAC-2 cells are mammary gland cells, cultured on collagen (Irminger-Finger et al., 1998). While cells of each cell type, when grown individually, showed adherent cells, the co-culture of CTBs and TAC-2 cells resulted in detachment of TAC-2 cells (Fig. 13A). Immunofluorescence staining of fixed cells showed that at low density of CTBs, TAC-2 cells were growing around CTBs and were not making contact with CTBs, when co-

cultured (Fig. 13B). Since CTBs produce MMPs (Cohen et al., 2006) that degrade matrix proteins like collagen, one explanation for TAC-2 cells to detach when co-cultured with CTBs, could be the degradation of the collagen matrix.

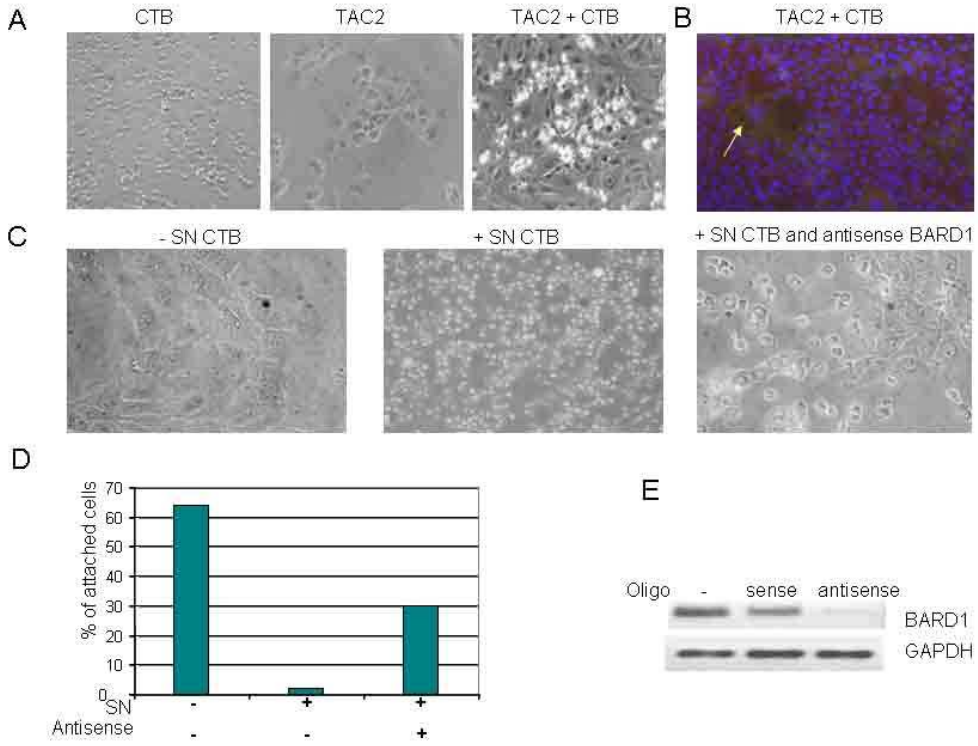


Figure 13. **Influence of CTB secretion on adhesion of collagen-dependent TAC2 cells.** (A) Co-culture of CTB and TAC2 cells shows detachment of TAC2 cells. (B) Immunofluroscence of co-culture of CTB and TAC2 cells shows growth inhibition of TAC2 cells around CTBs (arrow). (C) TAC2 cells cultured with or without supernatant of CTB, and supernatant of CTB transfected with inhibitory BARD1 antisense oligos. (D) Histogram of TAC2 cells cultured with or without supernatant of CTB, and supernatant of CTBs transfected with BARD1 antisense oligos. (E) Effect of BARD1 sense and antisense oligos transfection on BARD1 expression in HeLa cells tested by RT-PCR. Control RT-PCR of GAPDH of the same samples is shown.

To investigate whether BARD1 was involved in this process, we tested whether repression of BARD1 expression in CTBs influenced their effect on TAC2 cell attachment. TAC2 cells were cultured with or without supernatants of CTBs, either transfected with BARD1 antisense or sense oligos, or control CTBs. After 24 hours, almost all TAC2 cells detached when exposed to supernatant from CTBs, and only few cells survived after 48 hours in cell cultures treated with the supernatant from sense oligo transfected or untransfected CTBs. When supernatants from CTBs transfected with BARD1 antisense oligos were applied, 30 percent of

TAC2 cells remained attached after 24 hours and 20 percent survived after 48 hours (Fig. 13C and D). Thus, inhibiting BARD1 expression in CTBs reduced the effect of CTBs on TAC2 cells adhesion. Since CTBs are not proliferating in culture and they are limited patient material, we performed parallel transfections with the same oligos in HeLa cells and performed RT-PCR to monitor BARD1 expression levels (Fig. 13E).

3. BARD1 expression in cancer cells

From the observations above, we found that BARD1 and spliced isoforms were expressed in CTBs of early pregnancy, and might play a role in proliferation and invasion of CTBs. Therefore, it was interesting to investigate further whether BARD1 isoforms were expressed in human cancers.

3.1. Different expression pattern of BARD1 isoforms in different cancer cell lines

First we performed RT-PCR on RNA from different gynaecological cancer cell lines to characterize BARD1 expression. We used primers located in various exons of BARD1 to amplify different regions of BARD1 in breast, cervical, endometrial, and ovarian cancer cell lines. We observed a specific BARD1 expression pattern of cell lines derived from different cancers. First in breast cancer cell lines FL BARD1 was expressed together with smaller isoforms β, φ, δ, and ε, which were more abundant than FL BARD1. Another group showed no expression at all when primers were used for amplification of FL BARD1 (Fig. 14A). In all cervical cancer cell lines, we found neither FL BARD1 nor isoforms expressed, when we performed RT-PCR to amplify exon 1 to exon 11. Then we used different forward primers more downstream to amplify potentially 5' truncated forms of BARD1, and we detected BARD1 expression when using primers at different sites in exon 4. Finally, BARD1

expression was found in all samples when we used forward primer in exon 5 (Fig. 15). It seems that these BARD1 isoforms were initiated in exon 4 in cervical cancer cell lines. In a control experiment, we tried to amplify the BARD1 region from exon 1 to exon 6, and there was indeed no transcript expression.

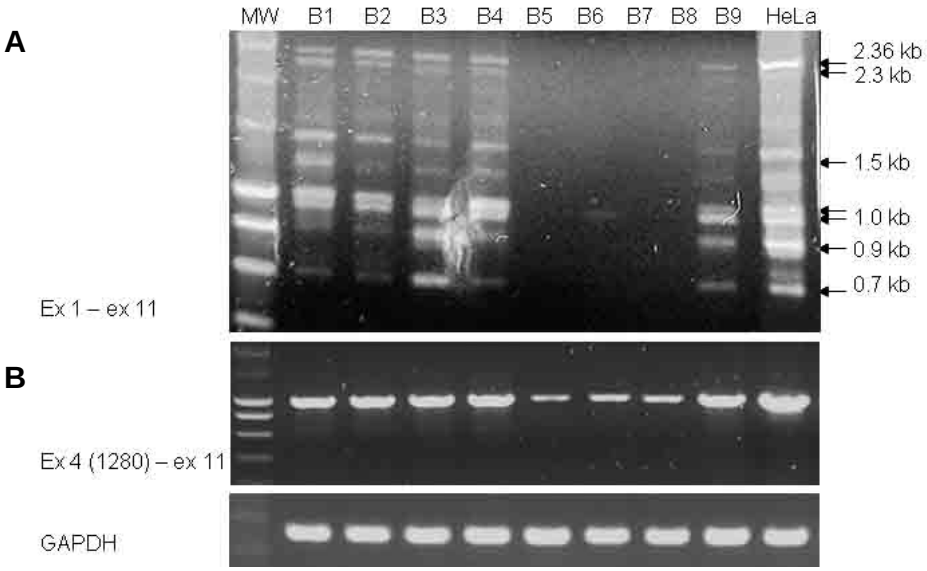


***Figure 14*. RT-PCR of breast cancer cell lines (B2-B9) for amplification of BARD1.** (A) Amplification of FL BARD1. (B) Amplification of FL BARD1 and truncated isoform from exon 4 through exon 11. HeLa cells were used as a control. GAPDH expression is shown in the same samples.

We performed RT-PCR in endometrial and ovarian cancer cell lines by using forward primers within exon 1 or exon 4 (Fig. 16A and B). FL BARD1 and isoforms were expressed in some of the samples. In other samples, which showed neither FL BARD1 nor isoforms, BARD1 was detected by forward primers within exon 4. When we performed RT-PCR on breast cancer cell lines in which showed no amplification with forward primer in exon 1, we detected BARD1 by forward primer in exon 4 (Fig. 14B).

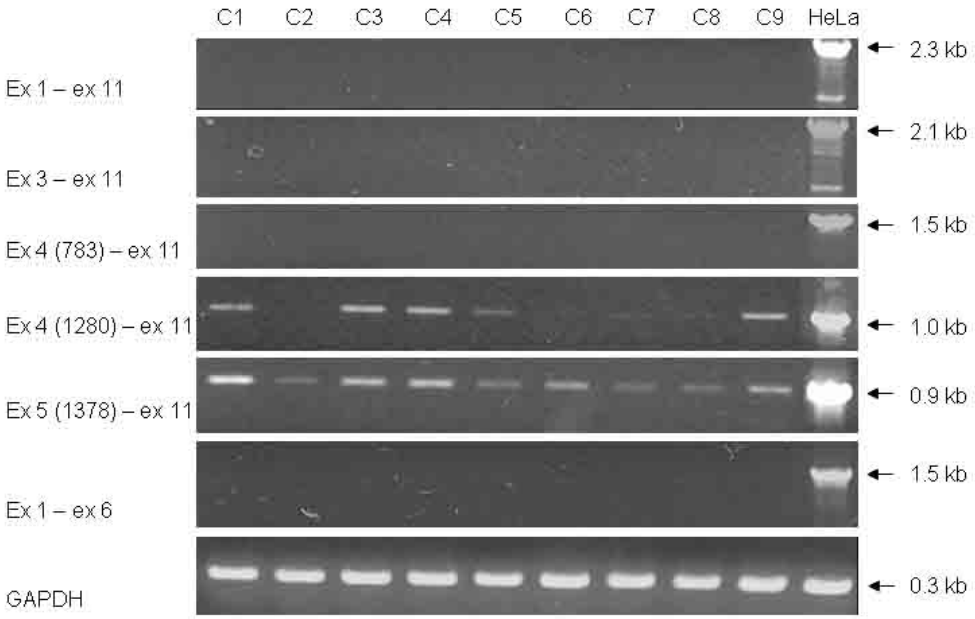


Figure 15. **RT-PCR of cervical cancer cell lines (C1-C9) for amplification of regions as indicated.** Primer positions are indicated referring to exons or nucleotide positions within exon 4 or 5, respectively. Amplicons were generated from HeLa cells in parallel. GAPDH expression is shown of the same samples.

In summary (Table 3), in none of the cervical cancer cell lines tested we found expression of FL BARD1, and BARD1 transcripts were only present from exon 4 through exon 11. In endometrial cancer cells, FL and spliced isoforms were expressed in 55.6% cases, in 11.1% spliced isoforms were present only, and 33.3% showed transcripts from exon 4 through exon 11. In breast cancer cells, 19.2% expressed FL BARD1 and isoforms, and most of the cell lines expressed BARD1 from exon 4, which accounted for about 80.8%. In ovarian cancer cell lines, 21.9% expressed FL and isoforms, 15.6% expressed spliced isoforms only, and 62.5% expressed transcripts comprising exon 4 through exon 11. All the cancer cell lines that we tested were derived from cancers that might be hormonally regulated. In all gynaecological cancer cell lines tested, FL BARD1 was either missing or it seemed less abundant than other spliced isoforms. However, in none of the cancer cell lines BARD1 expression was absent.

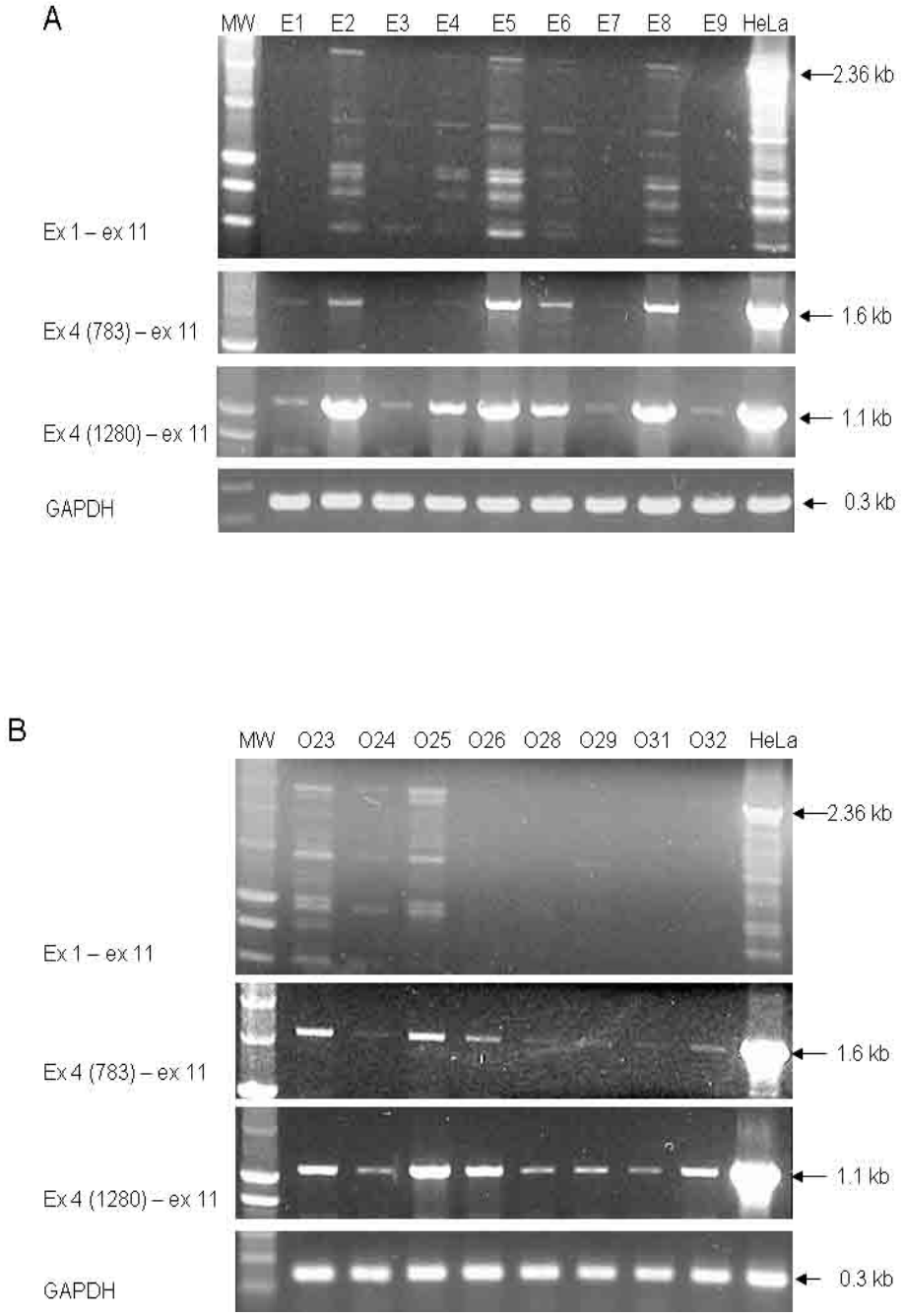


Figure 16. **Amplification of FL BARD1 and truncated isoform from exon 4 through exon 11 in endometrial (E1-E9) and ovarian (O23-O32) cancer cell lines.** (A) RT-PCR in endometrial cancer cell lines. (B) RT-PCR in ovarian cancer cell lines. HeLa cells were used as a control. GAPDH expression was tested in the same samples.

Table 3. BARD1 isoforms in different cancer cell lines.

Type of cancer	Full length only	Full length & spliced isoforms	Spliced isoforms only	New start in exon 4 only
Cervical cancer	0	0	0	100% (9/9)
Breast cancer	0	19.2% (5/26)	0	80.8% (21/26)
Ovarian cancer	0	21.9% (7/32)	15.6% (5/32)	62.5% (20/32)
Endometrial cancer	0	55.6% (5/9)	11.1% (1/9)	33.3% (3/9)
Haemotological cancer	100% (13/13)	0% (0/13)	0	Not determined

As a comparison, RT-PCR was also performed in haematological tumour cell lines which are unlikely to be hormonally controlled (Fig. 17). In this case, most of the samples showed FL BARD1 expression, and nearly no smaller spliced isoforms were shown. Thus, we conclude that FL BARD1 is often lost in gynaecological cancer cell lines, but isoforms are expressed. In addition to differently spliced isoforms, a 5' truncated form, comprising exon 4 through exon 11, is expressed.

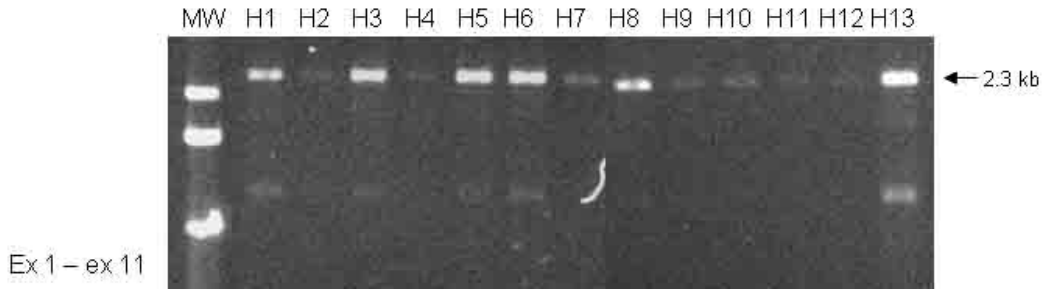


Figure 17. **RT-PCR of BARD1 expression in haematological tumour cell lines (H1-H13).** FL BARD1 was expressed and isoform γ was weekly detected.

3.2. No promoter silencing of BARD1 in ovarian cancer

DNA methylation in tumour suppressor genes is one of the mechanisms of carcinogenesis. Hypermethylation of BRCA1 was observed in sporatic breast cancer (Bianco et al., 2000; Dobrovic and Simpfendorfer, 1997). Since different mutations are various induced in cancer

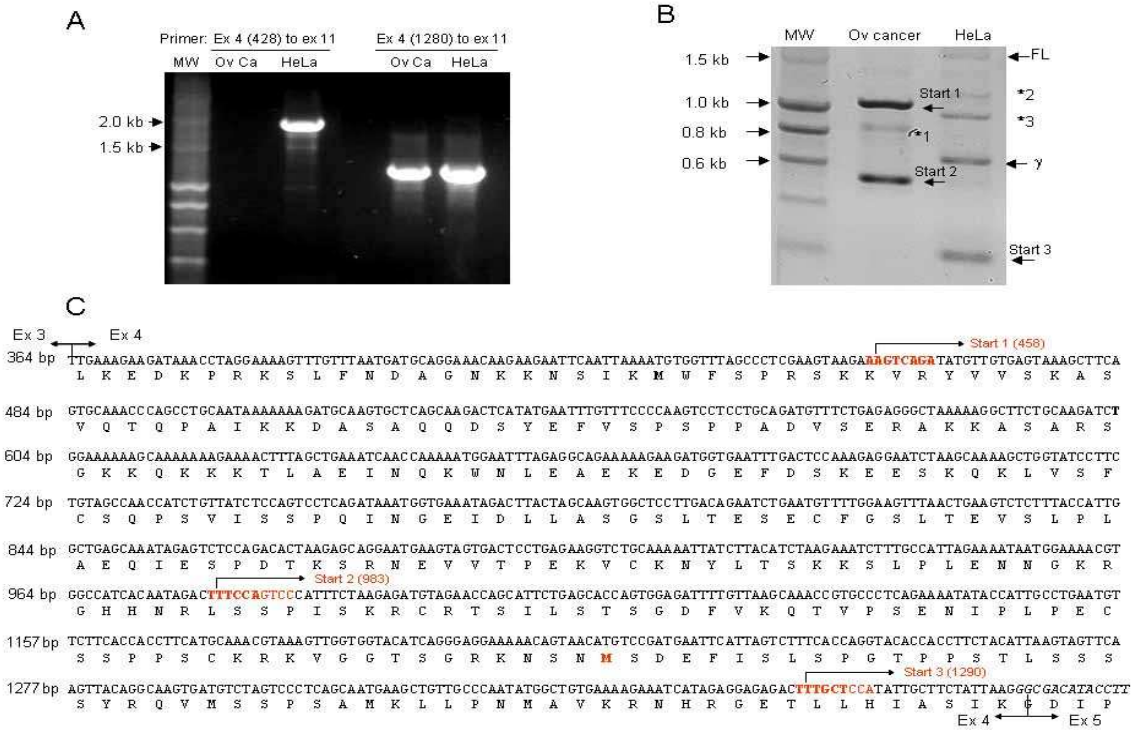
cells, we wondered whether BARD1 was also silenced. A panel of 50 primary epithelial ovarian cancer tissue samples and 34 ovarian cancer cell lines were analyzed for hypermethylation of the BARD1 promoter by methylation specific PCR. No evidence for hypermethylation of BARD1 in any primary tumour sample or cancer cell line in ovarian cancer could be detected, suggesting that BARD1 is an essential gene required for cell viability. It also suggests that isoforms expressed in cancer cells might encode essential functions for cancer growth.

3.3.Alternative initiation of transcription in exon 4

From the results described above, we suspected that transcripts comprising exon 4 to exon 11 might have an alternative site of transcription initiation. This is consistent with previous reports of loss of N terminal epitopes are found in ovarian cancer samples (Wu et al., 2006). Therefore we performed RT-PCR on ovarian cancer samples with N-terminal loss. We used forward primers located at the beginning of exon 4 (428) and the end of exon 4 (1280), respectively, but we only detected BARD1 expression with forward primer at the end of exon 4 (1280) (Fig. 18A).

Therefore, we performed 5' race experiments with 5' GeneRacer to amplify 5' cDNA ends, using RNA from ovarian cancer samples and HeLa cells to determine the initiation of transcription. After performing 5' PCR and nested PCR, we purified and sequenced the amplicons obtained (Fig. 18B). In HeLa cells, we found that two of the amplicons corresponded to FL BARD1 and isoform γ with the normal BARD1 initiation site, and one corresponded to a new transcription initiation within exon 4. However, we found two new transcription initiations within exon 4 in ovarian cancer. One was at the nucleotide position 458 (start 1), which corresponds to the beginning part of exon 4 and the other was at nucleotide 983 (start 2) in exon 4. In HeLa cells, the new transcription initiation was located

at the end of exon 4, at nucleotide position 1290 (start 3) (Fig. 18C). Transcription of start 1 and 2 transcripts initiates at the same ATG within exon 4 and produce a protein of approximately 44 kD, and start 3 transcripts could produce a protein of about 27 kD. The mRNA and translated sequence structure is shown in Figure 18C and D. We named the new isoforms initiating in exon 4 as Ω1, Ω2 and Ψ. The new transcription initiation sites are consistent with our results obtained by RT-PCR, which showed that the regions comprising exon 4 through exon 11, but not exon 1 to exon 3, were present in many samples. The forward primers that we used within exon 4 at nucleotide position 783 could detect isoform Ω1, and primers at nucleotide position 1280 detected isofom Ω1 and Ω2. Isoform Ψ could be detected by forward primer within exon 5 at nucleotide position 1378. In cervical cancer cell lines, Ω2 was shown in 5 of the nine samples, and Ψ was shown in the other 4 samples (Fig. 15). Interestingly, in endometrial and ovarian cancer cell lines, Ω1 or Ω2 were detected in those samples that have no expression of FL BARD1. These data demonstrated that gynaecological cancers express truncated BARD1 isoforms due to alternative transcription initiation in exon 4 or splice isoforms.



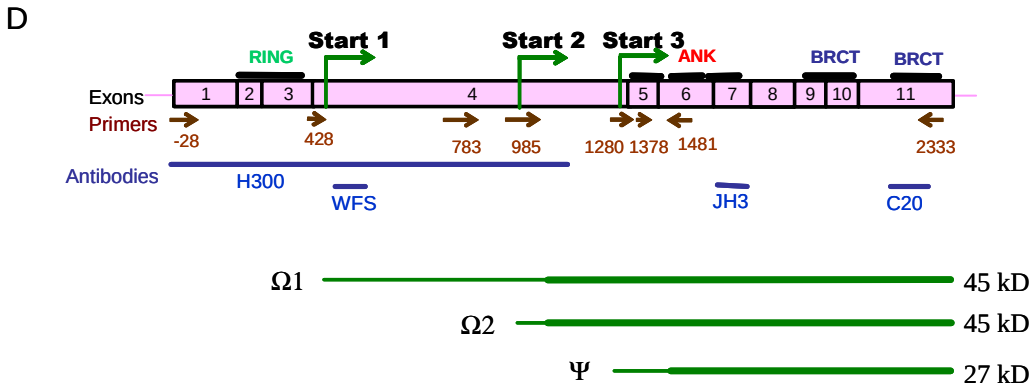


Figure 18. **Alternative initiation of transcription in exon 4.** (A) RT-PCR of ovarian cancer (Ov Ca) sample with forward primers within exon 4 (428 or 1280) and reverse primer in exon 11. (B) Nested PCR of 5' race experiment of ovarian cancer sample and HeLa cells. Forward primer was 5' nested primer and reverse primer located within exon 6. The bands sequenced are indicated by arrows. Band*1 indicates band with identical sequence as *start 1*, but different gel migration for unknown reason. Bands *2 and *3 were not sequenced. Transcript derived from new transcription initiations are indicated by arrows. (C) Protein and mRNA sequence of BARD1 exon 4. Positions of new initiations found by 5' race experiment are indicated. (D) Diagram of BARD1 exon structure and three isoforms derived from alternative transcript initiation. Primers and antibodies used in the experiments are shown. The translated regions were shown in thick lines, non-translated in thin lines.

3.4. Identification of protein isoforms in ovarian cancer cell lines

As we observed different BARD1 transcripts in cancer cells, we were interested in elucidating whether these isoforms were translated. We performed Western blots analysis on protein extracts from ovarian cancer cell lines (Fig. 19). HeLaBig cells, with exogenous overexpression of FL BARD1 were used as a control. We used BARD1 antibody H300 detecting epitopes expressed on exons 1 through 4 for N-terminus and antibody JH3 directed against a peptide antigen within exon 7 (Fig. 18D). When using H300, we found that FL BARD1, which is a 87 kD protein but migrates on the gel as a band of 97 kD, was detected in extracts from HeLaBig cells, but none of the ovarian cancer samples showed FL BARD1. We detected a protein band of 94 kD in some of the ovarian samples, and a 84 kD protein in all samples. Deducing from the cDNA structure of BARD1 isoforms (Fig. 5B), the 94 kD and 84 kD bands corresponded to isoform α (deletion exon 2) and isoform β (deletion exon 2 and 3), respectively, considered their migration on the gel like FL BARD1, they might be slightly higher than their calculated MW. In some of the samples, we observed two smaller bands of

about 40 kD, which might be isoforms φ (deletion of exon 3 to 6) and δ (deletion of exon 2 to 6). However, when probing with JH3, we detected a very strong band of 48 kD which was not detected by H300. This N-terminally truncated form was abundantly expressed in all ovarian cancer samples. The observed MW of this protein corresponds to the calculated MW (44 kD) of isoform Ω1 and Ω 2, which might be on the gel as a 48 kD protein. This 48 kD protein derived from isoform Ω 1 and Ω 2, and is consistent with our RT-PCR result. We also deduced that the other smaller band of about 41 kDa, detected by JH3 could be isoform φ or δ, which could also be detected by H300. These protein detections thus confirmed the results obtained by RT-PCR and made evident that there was little or no FL BARD1 expressed in ovarian cancer, but abundant expression of isoforms. Compared to the isoforms derived from differential splicing, Ω isoforms were much more abundant, which is similar to what we observed by RT-PCR (Fig. 16B).

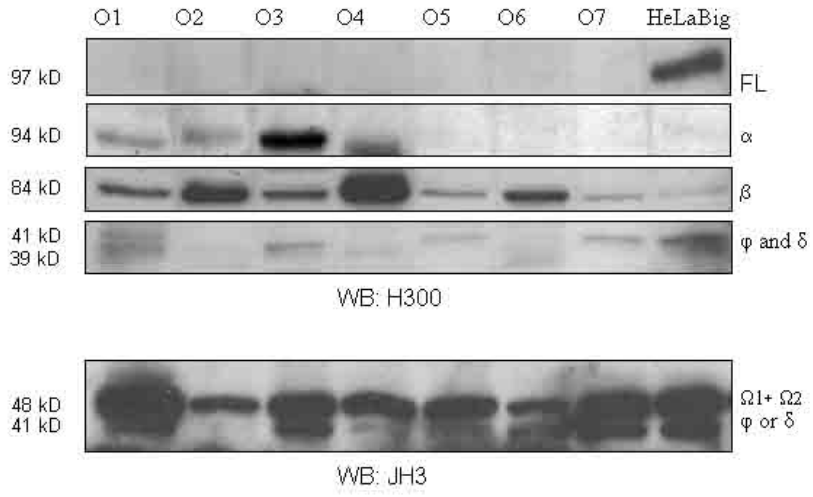


Figure 19. **Western blot analysis in ovarian cancer cell lines probed with BARD1 antibodies H300 and JH3.** MW of different BARD1 isoforms and presumed isoform identities are indicated. HeLa cells expressing exogenous BARD1 (HeLaBig) cells were used as a control.

3.5.BARD1 expression and clinical prognosis in cancer

To investigate how BARD1 is expressed *in vivo*, we performed immunohistochemical staining on different type of ovarian and colon cancers. Cancer samples were prepared as tissue micro array (Fig. 20). A total of 106 cases of ovarian cancer from women of 32-87 years old were analyzed. Different antibodies detecting epitopes at the N-terminus (N19), within exon 4 (WFS), and C-terminus (C-20) of BARD1 were used. We observed that antibodies N19 and C20 detected high expression of BARD1 in some of the patient samples, but weak in others. However, antibody WFS, which detects an epitope within exon 4 was expressed weakly in most of the cancer samples. We also found that the staining of N19 was lost or decreased in some of the samples, consistent with our previous finding (Wu et al., 2006). Interestingly, the loss of N19 expression mostly happened in ovarian cancer of T3 stage (Fig. 21A, C) or cancers with lymph node metastasis (N1) (Fig. 21B) ($p<0.05$). Thus N terminal loss is correlated with the advanced stage of ovarian cancer. However, we did not find a correlation of the tumour grade and BARD1 expression (Fig. 21D). Expressed C-terminus and loss of N-terminus correspond to the expression of Ω isoforms. Furthermore, we found that both N19 and C20, but not for WFS were highly expressed in clear cell carcinoma, which is the type of ovarian cancer with the worst prognosis (Fig. 22A, C). This expression pattern is consistent with the expression of isoform φ, δ and ε. RT-PCR performed in ovarian cancer cell lines derived from clear cell carcinoma confirmed this hypothesis. Elevated expression of isoforms φ, δ, and ε, but no FL BARD1 in SK-OV-3 and TOV-21G cell lines, which are of clear cell type, was found (Fig. 22B). From these data, we may conclude that expression Ω isoforms of BARD1 in ovarian cancer is correlated with advanced stage of ovarian cancer, and spliced isoforms with poor prognosis.

We performed immunohistochemical staining on 145 cases of colon cancer tissue micro arrays also. Different antibodies for the N-terminus (N19), within exon 3 (PVC), and C-

terminus (C-20) of BARD1 were used. We observed that there were different BARD1 expression patterns in these samples. BARD1 were highly expressed in some of the samples, but not in others (Fig. 23). But after further analysis, we did not find a correlation of BARD1 expression and tumour stage (Fig. 24).

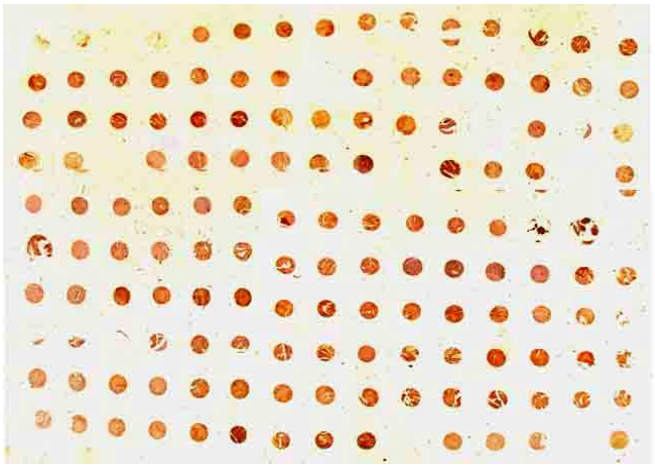
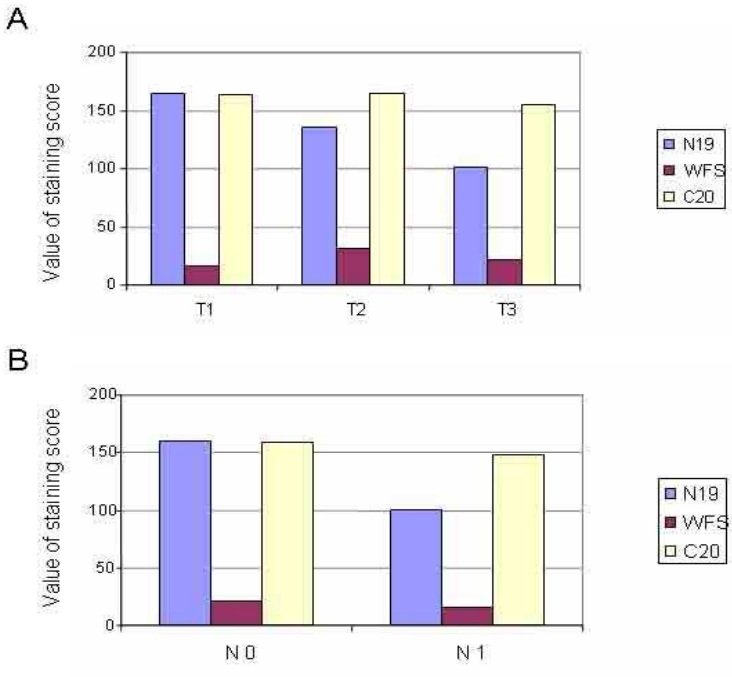
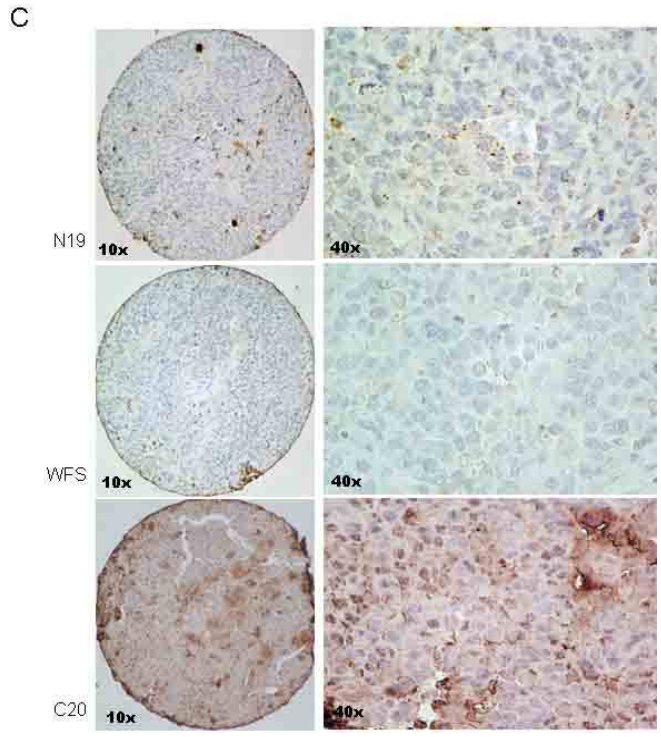


Figure 20. **Example of immunohistochemistry of tissue micro arrays.** Samples were presented in triplicates for each patient.





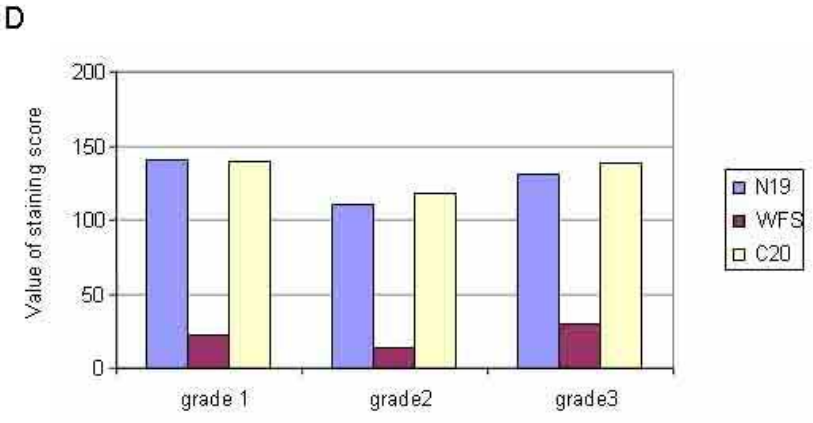


Figure 21. **Analysis of BARD1 expression in ovarian cancer using tissue arrays.** (A) Correlation of BARD1 expression and primary tumour stage. T1: Tumour is limited to one or both ovaries. T2: Tumour involves one or both ovaries with spread into the pelvis. T3: Tumour involves one or both ovaries, with microscopically confirmed peritoneal metastasis outside the pelvis and/or metastasis to regional (nearby) lymph node(s). (B) Correlation of BARD1 expression and lymph node metastasis. N0: Regional lymph nodes contain no metastases. N1: Evidence of lymph node metastasis. (C) Immunohistochemistry of one example of patient at stage T3 showing both N19 and WFS negative, but C20 strongly positive staining. (D) Correlation of BARD1 expression and different pathology grade in ovarian cancer. G1: the least malignant, with well-differentiated cells. G2: intermediate, with moderately differentiated cells. G3: the most malignant, with poorly differentiated cells.

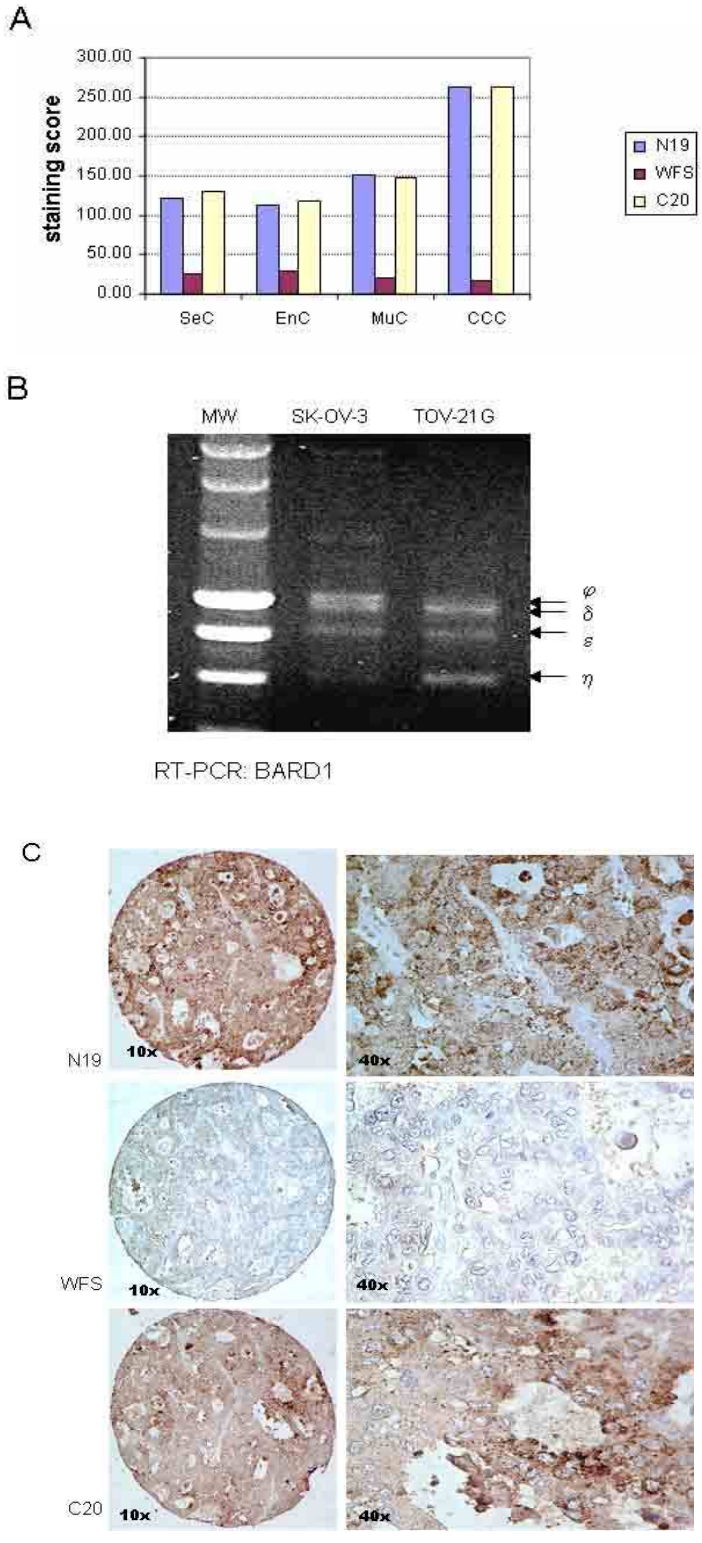


Figure 22. **BARD1 expression in different pathological types of ovarian cancer.** (A) Immunohistochemical staining in different pathologic types. Clear cell carcinoma has the highest score. SeC, serous carcinoma; EnC, endometriod carcinoma; CCC, clear cell carcinoma; MuC, mucinous carcinoma. (B) RT-PCR for amplification of FL BARD1 in clear cell carcinoma cell lines. (C) Immunohistochemistry of clear cell carcinoma showed strong staining with N19 and C20, but was negative by WFS.

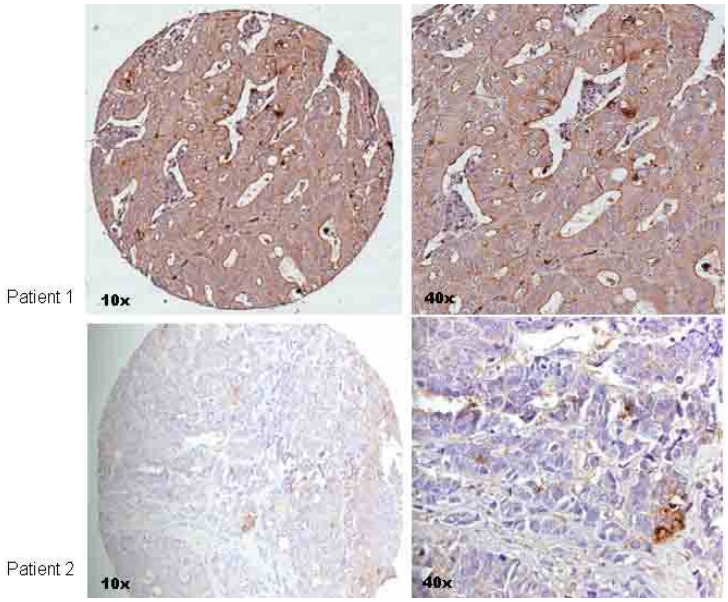


Figure 23. **Immunohistochemistry of BARD1 expression in colon cancer.** Examples of high BARD1 expression in patient 1 and negative expression in patient 2 are shown.

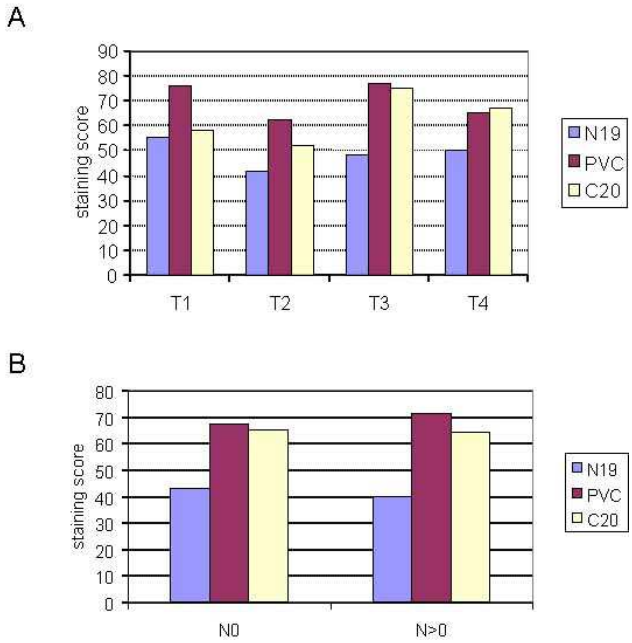


Figure 24. **Immunohistochemical staining score in colon cancer.** (A) BARD1 expression in colon cancer of different primary tumour stage. (B) BARD1 expression in colon cancer with or without lymph node metastasis. No correlation was found with different stages of colon cancer.

Discussion

BARD1 has been attracting much attention in the last few years, and evidence is accumulating that it is a tumour suppressor, both as a binding partner of the breast cancer protein BRCA1 and independently due to its presumed functions in tumorigenesis (Irminger-Finger and Jefford, 2006). Inherited predisposition to breast and ovarian cancer is associated in up to 50 percent of cases with mutations in BRCA1 and BRCA2. BARD1 mutations have been found in inherited and spontaneous cases of breast and ovarian cancer, but with very low frequency. Considering the multiple functions of BARD1 as a tumour suppressor, it could be expected that its functions might be lost or abrogated in cancer cells. It was therefore interesting and surprising to find that BARD1 was highly expressed in cancer cells, and mislocated to the cytoplasm, but not associated with apoptosis (Wu et al., 2006). However, in 70% of ovarian cancer samples, only the C-terminal portion of the coding region was present when amplified by RT-PCR (Wu et al., 2006). This is consistent with the notion that there are aberrant transcripts in cancer. In addition, several alternatively spliced transcripts, but no full length BARD1, were found in a rat ovarian caner cell line, which is resistant to apoptosis (Feki et al., 2004), suggesting a potential role of BARD1 isoforms in cancer. We hypothesized that specific isoforms of BARD1 might have lost tumour suppressor functions and acquired tumorigenic properties. Therefore, we were interested in elucidating the functions of BARD1 isoforms in cancer cells further to determine the functional roles of these splice variants.

Human cytotrophoblasts share high similarity with cancer cells in the process of proliferation and invasion process. From the data presented here, we know that BARD1 expression is highly elevated in CTBs of early pregnancy, as compared to other tissues, suggesting that it plays an important role in this cell type. BRCA1, however, is only weakly expressed in CTBs, indicating that the role of BARD1 in CTBs is independent of BRCA1.

1. Temporal and spatial expression of BARD1 and its isoforms in CTBs

In the first trimester of human pregnancy, the cytotrophoblasts proliferate and invade deep into the maternal decidua under strict temporal and spatial control, which is essential for the success of pregnancy (Cross et al., 1994; Morrish et al., 2001). We found that BARD1 and its differentially spliced isoforms were also expressed in first trimester of pregnancy in a temporally controlled manner. They are increased from 7 to 9 weeks and decreased thereafter, which suggests a role in the temporal control of invasiveness of CTBs. We found that BARD1 isoforms are expressed at this stage, and they are either deprived of the BRCA1-interacting RING finger domain, i.e. isoform β, δ, and η, or lost the ANK region, like isoform ε, which suggests that their functions are different from FL BARD1.

The expression profile of BARD1 in early pregnancy parallels hCG levels in the blood, known to increase and peak at the time of cytotrophoblast invasion and to decrease after the 12^{th} week of pregnancy (Chartier et al., 1979). In purified CTBs, the mRNA levels of the inducible form of hCG, β-hCG, increase and decrease parallel to BARD1 mRNA levels, thus confirming a correlation of time of expression of BARD1 and hCG. Indeed *in vitro* experiments demonstrate that hCG is both a transcriptional and translational inducer of BARD1 expression, since BARD1 isoforms were especially upregulated by hCG.

While the temporal regulation of BARD1 expression can be explained by a regulatory function of hCG, it is also consistent with the local variation of oxygen levels in the placenta during the first trimester. It is believed that the hypoxic environment of the first trimester plays an important role in placentation (Graham et al., 2000); low oxygen levels can upregulate invasiveness of trophoblasts and of other cell types, such as human breast carcinoma cells (Graham et al., 1999). We found that BARD1 and its spliced isoforms were also upregulated by hypoxia. Since hCG and hypoxia can upregulate invasiveness of CTBs, and they both induce BARD1 expression in first trimester CTBs, we hypothesized that

BARD1 and/or its isoforms play a role in invasiveness of CTBs in first trimester of pregnancy.

Indeed, we found that expression of BARD1 isoforms was spatially controlled. Isoform δ was expressed in villous CTBs, which have a proliferative function like stem cell (Maltepe et al., 2005). Isoform ε is expressed in extravillous CTBs, which are the cells that invade into the maternal decidua. Within the extravillous CTB cell columns, isoform δ is expressed in the proliferation region and isoform ε in the invasive region, indicating a precise spatial regulation of specific isoform expression pattern. Therefore, we conclude that differentially spliced isoforms of BARD1 in first trimester placenta are expressed in a spatially and temporally regulated manner and might account for distinct functions, such as isoform δ for proliferation and isoform ε for invasion.

2. BARD1 isoforms were secreted by CTBs

BARD1 expression in CTBs was found mostly in the cytoplasm, while in proliferating cell cultures it is observed as a nuclear protein. Furthermore, we found that isoforms that are compatible with BARD1β and ε, based on the size of the protein bands on Western blots and antibody detection, were secreted by CTBs. Indeed, we could easily detect BARD1 in cell supernatant of CTBs but not in supernatants of other cell lines, and Western blots analysis suggests that isoform β and ε are secreted in the supernatant of CTBs. The feature of cytoplasmic localization and secretion of nuclear proteins by non-canonical pathways is often found in cancer. One prominent example is the high mobility group protein (HMGB1), a chromatin component, which is localized to the cytoplasm and secreted in cancer (Lotze and Tracey, 2005). Thus, secreted isoforms of BARD1 might have a role in early trophoblast invasion and one of the mechanisms might be remodelling of the extracellular matrix.

Since both hCG and hypoxia are factors implicated in control of the invasive capacities of CTBs (Bischof and Irminger-Finger, 2005), BARD1 could be a mediator in a pathway of regulating CTB invasion. Invasion potential strongly depends on the production and secretion of MMPs by CTBs (Bischof and Irminger-Finger, 2005). Indeed the supernatant of CTBs cell cultures is sufficient to disturb attachment of epithelial cells that need collagen for their growth. More interestingly, repression of BARD1 in these CTBs, reduces the effect of the supernatant on epithelial cell attachment. This observation could be explained by either an effect of BARD1 on cell adhesion or on matrix proteolysis by MMPs.

The present observations would be consistent with a phenotype of infertility of the BARD1 knock-out mouse (McCarthy et al., 2003). However, reduced fertility of the BARD1 knock-out mice was not investigated, and if observed, might have been contributed to the embryonic lethality of the offspring. Future investigations should therefore take advantage of a directed repression of CTB-specific isoforms to produce phenotypes that permit to confirm this hypothesis.

All characteristics of BARD1 expressed in CTBs, such as the intracellular localization to the cytoplasm but not to the nucleus and the function in proliferation and invasion, are similar to the observations made in cancer cells, where truncated forms of BARD1 are highly upregulated and localized to the cytoplasm. It was therefore interesting to determine whether BARD1 isoforms that play a role in CTB proliferation and invasion exist in tumours and contribute to their progression. Therefore, the functional analysis of BARD1 isoforms in CTBs was paralleled by studying their expression in cancer cells.

3. Expression of BARD1 isoforms in cancers

In HeLa cells we found isoform α, β, γ, δ, φ, ε, and η. All of them are likely to code proteins, except for isoform γ, which does not have continuous open reading frame. Our observations

on various types of gynaecological cancer cell lines suggest that a specific pattern of BARD1 isoform expression exists in different cancers (Table 3). Compared to the normal tissue, in which FL BARD1 is highly expressed, there was little FL BARD1 in cancer cell lines, but BARD1 isoforms were abundantly expressed. In breast, ovarian and endometrial cancer, spliced isoforms of BARD1 were expressed. These isoforms are either deprived of the BRCA1-interacting RING finger domain, i.e. isoform β, δ, φ and η, or had lost the ANK or part of BRCT region, like isoform ε and η, which suggests loss of tumour suppressor functions. In addition, N terminal truncated forms were found in all gynaecological cancer cell lines, especially in all samples of cervical cancer cell line. These truncated isoforms are due to a new initiation of transcription within BARD1 exon 4. Such transcripts can be translated into a 44 kD protein, and are highly expressed in gynaecological cancers, while FL BARD1 was absent. Western blot analysis in ovarian cancer cell line samples, confirms that there was no expression of FL BARD1, however, different isoforms of BARD1 were highly expressed. Especially, isoform Ω was abundantly expressed in ovarian cancer.

BARD1 isoforms seem to be specifically expressed in gynaecological cancers, since FL BARD1, but none of the described isoforms were found in haematological cancer cell lines. Therefore, we concluded that BARD1 transcript and protein isoforms are specifically expressed in gynaecological cancers.

It was interesting that in all cancer cell lines tested, we could always detect BARD1 isoforms or FL, but never find cells that are avoid of BARD1. In order to further test of this notion, we proceeded methylation assays on 50 primary ovarian cancer samples and 34 ovarian cancer cell lines, and confirmed that there was no promoter silencing in these cancers. From this we can conclude that BARD1 isoforms might be essential for cancer cell growth. Cancer specific BARD1 isoforms might have lost tumour suppressor functions, but retained or acquired functions that play a functional role in tumour growth and/or invasion.

4. BARD1 isoforms and clinical prognosis

BARD1 expression studies by immunohistochemistry in 106 ovarian cancers confirmed the expression of aberrant BARD1 isoforms in cancer cells. A loss of N-terminus but not C-terminus was found in many of the samples, which is consistent with our previous finding from our lab on a smaller sample number (Wu et al., 2006). Weak expression of WFS (exon 4) was found in all samples, indicating that FL BARD1 was almost not expressed. The absence of N-terminal epitopes corresponds to the expression of isoform Ω. The lowest score of N19 (N-terminus) staining together with weak staining of WFS (exon 4) was found in T3 (tumour invades tissues outside of the pelvis) and N1 (positive for lymph node metastasis) stage, suggesting that isoform Ω might be more expressed in advanced stage of ovarian carcinoma. Thus, a mixture of BARD1 isoforms might be expressed in ovarian cancer of different stages. Since N-terminal loss was more frequent in advanced stage of ovarian cancer, isoform Ω might be a negative prognostic factor in ovarian cancer.

In addition, we observed a specific pattern of BARD1 expression in clear cell ovarian carcinoma, where strong staining of both N-19 and C-20, but not for WFS (exon 4) was found. This is consistent with expression of isoform φ, δ and ε in clear cell carcinoma, as identified by RT-PCR. Clear cell carcinoma is known as the type of ovarian cancer with poor prognosis and poor response to platinum-based chemotherapy (Goff et al., 1996). Interestingly, these isoforms are also found in CTBs. Isoform δ (similar to φ) expressed in proliferate CTBs, isoform ε in invasive CTBs (Li et al., 2007). Therefore, we hypothesized that there might be different patterns of BARD1 isoforms expressed at different stages of cancer, and also in cancers of different origin. Isoform Ω might be more expressed at the advanced stage of cancers, isoforms φ, δ, and ε, in association with loss of FL BARD1, are typical for clear cell carcinoma, the ovarian cancer with the worst prognosis.

In comparison, immunohistochemical staining in colon cancer did not show a correlation of BARD1 expression and cancer stage. Indeed, there were different expression patterns in these colon cancer samples. BARD1 was highly expressed in some cases, but negative in others. Therefore, the question arises: Does BARD1 play a more important role in hormone related cancers? Interestingly, a recent report suggests that ERα is a substrate for the BRCA1-BARD1 ubiquitin ligase. This provides a potential link between the loss of BRCA1-BARD1 ligase activity and tissue-specific carcinoma (Eakin et al., 2007). Another finding demonstrates that there is a region of the BARD1 gene within the ninth intron which confers estrogen responsiveness, and BARD1 mRNA and protein levels can be increased by estrogen (Creekmore et al., 2007). Taken together, BARD1 isoforms might play a role in cancer development and progression through an estrogen-regulated pathway.

To summarize, isoforms of BARD1 are more expressed than FL BARD1 in gynaecological cancers, and these isoforms lack tumour suppressor functions but might have acquired novel functions in favour of tumour growth. Specially, some of these isofoms might be a negative prognostic factor for ovarian cancer.

Ongoing work in our lab is focused on investigating the cellular function of BARD1 isoforms. Therefore, we transducted NuTu cells, a rat ovarian cancer cells, which lack FL BARD1 (Feki et al., 2004), but express isoform β and δ, with BARD1 siRNA to inhibit BARD1 expression. We observed a complete proliferation stop, and cell death, when siRNA targetting isoforms were applied (Data not shown). This is in line with recent work from our lab, which demonstrated an essential function of BARD1 in mitosis (Jefford, et al., submitted). These data provide an explanation for the finding of specific isoforms, but not FL BARD1 in cancers.

Conclusion

1. FL BARD1 and isoforms in CTBs in first trimester of pregnancy are expressed in a temporally and spatially controlled way. FL BARD1 and isoforms expression is regulated by hCG and hypoxia; both factors play a role in invasiveness. Specifically, isoform δ is expressed in villous and proliferative CTBs, isoform ε is expressed in invasive CTBs.
2. The supernatant of CTBs cell cultures is sufficient to disturb attachment of epithelial cells that need collagen for their growth, which can be due to an effect of BARD1 on cell adhesion or on matrix proteolysis by MMPs. At least two isoforms of BARD1 are secreted by CTBs, and may play a role in extracellular matrix remodelling and trophoblasts invasion.
3. FL BARD1 is expressed in normal cells but not or less in cancer cells, while isoforms are more expressed in cancer cells. There seems to be a specific pattern of isoform expression in different types of cancer.
4. While spliced isoforms are typically found in breast, ovarian, endometrial cancer, a new initiation of transcription within exon 4, producing a truncated isoform Ω comprising of BARD1 exon 4 through 11, was found.
5. In a large collection of ovarian cancer (106 patients), BARD1 isoforms are expressed, but not FL BARD1. N-terminal epitopes loss, compatible with expression of isoform Ω, is associated with advanced stage of cancer. Expressions of isoforms φ, δ and ε, but not FL BARD1, were associated with clear cell carcinoma, linking these isoforms to the worst prognosis.
6. Future: Can BARD1 isoforms be tumour markers? Can BARD1 isoform be targets for new strategies for cancer treatment?

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Appendix 1.

Manuscript published: (accepted and in press in *International Journal of Biochemistry and Cell Biology*)

Identification of BARD1 splice-isoforms involved in human trophoblast invasion

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Appendix 2.

Manuscript submitted:

Oncogenic BARD1 isoforms expressed in gynaecological cancers

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