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Application of Next Generation Sequencing in Mammalian Embryogenomics: Lessons Learned from Endogenous Betaretroviruses of Sheep

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Abstract

Endogenous retroviruses (ERVs) are present in the genome of all vertebrates and are remnants of ancient exogenous retroviral infections of the host germline transmitted vertically from generation to generation. The sheep genome contains 27 JSRV-related endogenous betaretroviruses (enJSRVs) related to the pathogenic Jaagsiekte sheep retrovirus (JSRV) that have been integrating in the host genome for the last 5 to 7 million years. The exogenous JSRV is a causative agent of a transmissible lung cancer in sheep, and enJSRVs are able to protect the host against JSRV infection. In sheep, the enJSRVs are most abundantly expressed in the uterine epithelia as well as in the conceptus (embryo and associated extraembryonic membranes) trophoblast. Sixteen of the 27 enJSRV loci contain an envelope (env) gene with an intact open reading frame, and in utero loss-of-function experiments found the enJSRVs Env to be essential for trophoblast outgrowth and conceptus elongation. Collectively, available evidence supports the ideas that genes captured from ancestral retroviruses were pivotal in the acquisition of new, important functions in mammalian evolution and were positively selected for biological roles in genome plasticity, protection of the host against infection of related pathogenic and exogenous retroviruses, and a convergent physiological role in placental morphogenesis and thus mammalian reproduction. The discovery of ERVs in mammals was initially based on molecular cloning discovery techniques and will be boosted forward by next generation sequencing technologies and *in silico* discovery techniques.

Keywords

sequencing; retrovirus; endogenous; placenta; uterus; sheep

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1. Endogenous retroviruses

Endogenous retroviruses (ERVs) are present in the genome of all vertebrates and are vertically transmitted as stable, inherited Mendelian genes (Boeke and Stoye, 1997). ERVs are thought to arise from ancient infections of the germline of the host by exogenous retroviruses. The obligatory integration step of the retroviral replication cycle allowed, during evolution, the incorporation of the viral genome (provirus) into the host genome (Fig. 1). Retrotransposition or re-infection of the germline can generate further insertions augmenting the number of ERVs loci in the genome (Gifford and Tristem, 2003). ERVs have heavily colonized the genome of all animal species; for example, they account for approximately 8% of the human genome (Lander et al., 2001) and 18% of the cattle genome (Adelson et al., 2009).

A complete ERV “provirus” (i.e. the retroviral genome integrated into the host cell genome) shares the same genomic structure of an exogenous retrovirus, which is four viral genes (*gag*, *pro*, *pol*, and *env*) flanked by two long terminal repeats (LTRs) (Fig. 2). The *gag* gene encodes for the major viral structural protein, while *pro* and *pol* encode for the viral enzymatic machinery necessary for the viral replication cycle. The *env* gene encodes for the envelope glycoprotein (Env) that is inserted in the lipid bilayer of the exterior membrane to form the viral envelope and mediates entry of the virus into susceptible cells. The LTRs contain enhancer and promoter elements that direct expression of the viral genes. Most ERVs are destined to extinction if their expression brings deleterious consequences for the host. Thus, their persistence in the host genome is the result of a fine balance reached throughout evolution which usually renders them replication defective due to the accumulation of mutations, deletions, rearrangements, and methylation (Boeke and Stoye, 1997).

ERVs are widespread throughout vertebrate genomes (Herniou et al., 1998). Some ERVs are highly related to exogenous retroviruses, including Jaagsiekte sheep retrovirus (JSRV), mouse mammary tumor virus (MMTV), feline leukemia virus (FeLV) and avian leukemia virus (ALV), which are currently active and infect sheep, mice, cats and chickens, respectively (Boeke and Stoye, 1997). These ERVs are generally referred to as “modern” ERVs, because of integration into the host genome after speciation and are closely related to exogenous viruses that are still infectious while most ERVs do not have an exogenous counterpart. Modern ERVs can also have insertionally polymorphic loci, since they are not completely fixed in a particular population and are still undergoing endogenization. For instance, both koalas and sheep are currently being invaded by the koala retrovirus (KoRV; Tarlinton et al., 2006) and endogenous JSRVs (enJSRVs; Arnaud et al., 2007b; Chessa et al., 2009), respectively. Indeed, some modern ERVs, such as enJSRVs, are still able to produce infectious virus due to the lack of inactivating mutations (Black et al., 2010). In contrast, “ancient” ERVs invaded the genomes before speciation and, consequently, are present in every individual at the same genomic location of phylogenetically related species (Coffin, 2004).

The biological significance of ERVs was debated for several decades, because these were generally thought to be “junk DNA” (Bock and Stoye, 2000). However, recent studies suggest that ERVs have a variety of beneficial roles to their host (Jern and Coffin, 2008; Varela et al., 2009; Kurth and Bannert, 2010). At the very least, the abundance of these elements in the host genome suggests that they contribute to genome plasticity. Moreover, the presence of transcriptionally active ERVs with intact open reading frames (ORFs) conserved million of years after integration supports the idea that some ERVs were exapted by the host for specific biological roles.

2. JSRV and enJSRVs of sheep

Domestic sheep harbor ERVs in their genome, termed enJSRVs, because they are highly related to the exogenous and pathogenic Jaagsiekte sheep retrovirus (JSRV) (Palmarini and Fan, 2003). JSRV is the causative agent of ovine pulmonary adenocarcinoma, a transmissible lung cancer of sheep (Palmarini et al., 1999). A unique feature of JSRV among oncogenic retroviruses is that its Env glycoprotein is the main determinant of cell transformation both *in vitro* and *in vivo* (Maeda et al., 2001; Rai et al., 2001; Allen et al., 2002; Zavala et al., 2003; Liu and Miller, 2005; Wootton et al., 2005; Caporale et al., 2006). Expression of the JSRV Env alone is able to transform a variety of cell lines *in vitro*, including mouse, rat and chicken fibroblasts as well as human bronchial, canine, and rat epithelial cells (Rai et al., 2001; Allen et al., 2002; Danilkovitch-Miagkova et al., 2003; Zavala et al., 2003; Liu and Miller, 2005; Varela et al., 2006). More importantly, the JSRV Env is able to induce lung adenocarcinomas in immunocompetent sheep when expressed by a JSRV-based vector under the control of the JSRV LTR (Caporale et al., 2006). Thus, JSRV Env is a dominant oncoprotein, however, the mechanisms of cell transformation induced by the JSRV Env are not completely understood. Although the mitogen activated protein kinase (Ras-MEK-MAPK), Rac1 and phosphoinositide 3-kinase (PI3K-AKT-mTOR) pathways are implicated in JSRV-induced cell transformation, it still remains to be determined how the cytoplasmic tail engages the cell signaling network to activate these pathways (Palmarini et al., 2001b; Maeda et al., 2005; De Las Heras et al., 2006; Varela et al., 2006; Maeda and Fan, 2008).

A series of studies over the past 20 years found that sheep genomes harbour many copies of enJSRVs (York et al., 1992; Hecht et al., 1996; Palmarini et al., 2000a; DeMartini et al., 2003; Arnaud et al., 2007b). Southern blot hybridization studies of genomic DNA indicated that their genome contained at least 15 to 20 copies of enJSRVs (York et al., 1992; Hecht et al., 1996). Molecular cloning studies, utilizing degenerate primer PCR and lambda phage genomic DNA library screening, identified three enJSRV proviruses of sheep (Palmarini et al., 2000a). Next, screening of a sheep genomic bacterial artificial chromosome (BAC) library, derived from DNA collected from a single Texel ram, found 27 copies of enJSRVs that were completely sequenced and characterized (Fig. 2) (Arnaud et al., 2007b). The majority of the 27 enJSRV proviruses were defective as a result of deletions, nonsense mutations, and recombinations; however, five enJSRV proviruses contain intact genomes with uninterrupted ORFs for all the retroviral genes (Arnaud et al., 2007b). JSRV and the enJSRVs have an overall high degree of similarity (~85–89% identity at the nucleotide level). The evolutionary history of these proviruses together with ruminants suggest that integration of enJSRVs began before the split between the genus *Ovis* and the genus *Capra*, approximately 5 to 7 million years ago, and continued after sheep domestication (~10,000 years ago) (Arnaud et al., 2007b; Chessa et al., 2009). Of note, enJSRVs are present in sheep and goats, but not cattle or other mammals. Interestingly, one enJSRV provirus, enJSRV-26, is thought to have integrated in the host less than 200 years ago and may be a unique integration event occurred in a single animal (Arnaud et al., 2007b).

Sheep and goats were the first livestock species to be domesticated (Ryder, 1983). Multiple domestication events, as inferred by multiple mitochondrial lineages, gave rise to domestic sheep and similarly other domestic species (Pedrosa et al., 2005; Tapio et al., 2006; Meadows et al., 2007; Naderi et al., 2008). Most enJSRVs loci are fixed in domestic sheep but some are differentially distributed between breeds and individuals (i.e., they are insertionally polymorphic) (Arnaud et al., 2007a; Chessa et al., 2009) (Fig. 3). Thus, enJSRVs can be used as highly informative genetic markers because the presence of each ERV in the host genome is the result of a single integration event in a single animal and is irreversible, so populations sharing the same provirus in the same genomic location are *de*

facto phylogenetically related. The study by Chessa and coworkers (Chessa et al., 2009) concluded that sheep, differentiated on the basis of their “retrotype” and morphological traits, dispersed across Eurasia and Africa via separate migratory episodes. That study provides genetic support to the theory that specialized wool production arose in South-West Asia and then spread throughout Europe (Sherratt, 1981).

3. Viral interference by enJSRVs

enJSRVs can block JSRV replication at both early and late stages of the retroviral cycle. Both JSRV and enJSRVs use the same cellular receptor for entry called HYAL2 (hyaluronoglucosaminidase 2), a glycosylphosphatidylinositol (GPI)-anchored protein. Thus, enJSRVs Env could prevent JSRV entry by a classic mechanism of receptor interference as described for other viruses (Spencer et al., 2003). In addition, two enJSRV loci (enJS56A1 and enJSRV-20) can block JSRV replication at a late stage of the retroviral replication cycle by a block referred to as JSRV late restriction (JLR) (Mura et al., 2004). These two transdominant proviruses entered the host genome 3 million years ago before and during speciation within the genus *Ovis*. They subsequently acquired in two temporally distinct events a defective Gag polypeptide via a substitution of an arginine at the position 21 (typical of a replication competent virus) to a tryptophan residue. JLR likely occurs via the production of defective Gag proteins by the transdominant proviruses that form viral particles and/or multimers with the functional Gag proteins, which then accumulate in the cytoplasm as pre-aggregate structures that are subsequently degraded by the proteasome. Therefore, the transdominant proviruses prevent Gag proteins of the competent virus to interact with the trafficking cellular machinery and ultimately the release of viral particles (Arnaud et al., 2007c; Murcia et al., 2007).

Available evidence strongly supports the hypothesis that selection of transdominant enJSRV loci protected sheep against infection with related exogenous retroviruses, including JSRV and perhaps enzootic nasal tumor virus or ENTV. Both proviruses with transdominant (protective) phenotypes became fixed in the host genome of the domestic sheep (*Ovis aries*) supporting the idea of their positive selection during or immediately before sheep domestication (9,000 y ago) (Arnaud et al., 2007b). These observations highlight the idea that some enJSRVs act as restriction factors and were selected during sheep domestication, supporting the hypothesis that ERVs could help the host to fight retroviral infections and might be useful to genetically engineer animals that are resistant to exogenous retroviruses.

4. ERVs and placental development in humans and mice

ERVs have been speculated to play a physiological role in placenta morphogenesis for almost three decades considering that retroviral particles have been frequently observed in the reproductive tract (Kalter et al., 1975; Smith and Moore, 1988; Harris, 1991). In fact, ERVs are abundant in the genital tract and placenta of various animal species (Harris, 1991; 1998). A number of intact ERV *env* genes have been identified in primates (syncytin-1 and -2) (Venables et al., 1995; Blond et al., 2000; de Parseval et al., 2003), Muridae (syncytin-A and -B in mouse, rat, gerbil, vole, and hamster) (Dupressoir et al., 2005a), rabbits (syncytin-Ory1) (Heidmann et al., 2009), and guinea pigs (Vernochet et al., 2011). In each case, the protein products of the nonorthologous ERV *env* genes, termed syncytins, are highly fusogenic in transfection assays and preferentially expressed in the syncytiotrophoblast. The syncytiotrophoblast is a multinucleated cell that lines the outer surface of the placenta, derived by intercellular fusion of mononuclear cytotrophoblast cells, and responsible for the transport of oxygen, nutrients and waste products, production of hormones, and immune tolerance (Watson and Cross, 2005). In both humans and mice, one of the two syncytins (human syncytin-2 and mouse syncytin-B) is immunosuppressive and, rather unexpectedly,

the other (human syncytin-1 and mouse syncytin-A) is not, although both are able to induce cell-cell fusion (Mangeney et al., 2007). Syncytins play an important biological role in syncytiotrophoblast development in mice. *Syncytin-A* null mice die *in utero* due to failure of trophoblast cells to fuse and form syncytiotrophoblast layer I in the mouse placenta (Dupressoir et al., 2009), and *syncytin-B* null mice display impaired formation of syncytiotrophoblast layer II (Dupressoir et al., 2011). Given that some syncytins are immunosuppressive, they may play a role in fetomaternal tolerance, although this concept has not been mechanistically tested *in vivo* (Mangeney et al., 2007). The presence of intact *env* genes that are expressed in the multinucleated syncytiotrophoblasts of the placenta and preserved over thousands of years, together with the observation that they elicit fusion of cells *in vitro*, led to the speculation that ERVs play an essential role in placental development and were positively selected for a convergent fundamental role in the evolution of placental mammals and development of viviparity (Villarreal and Villarreal, 1997; Mi et al., 2000; Dupressoir et al., 2005b; Heidmann et al., 2009).

5. The sheep placenta, conceptus development, and enJSRVs

In sheep, the morula-stage embryo enters the uterus by Day 5 after mating and form a blastocyst by Day 6 that contains a blastocoele surrounded by a monolayer of trophectoderm (Guillemot, 1995; Spencer et al., 2004). By Day 9, the blastocyst hatches from the zona pellucida, develops into an ovoid conceptus by Day 12, and then begins to elongate (reaching 25 cm or more by Day 17). Elongation of the conceptus is critical for the production of interferon tau (IFNT), which is the pregnancy recognition signal needed to maintain progesterone production by the corpus luteum, and also for the onset of implantation (Spencer et al., 2007). Implantation of the conceptus involves the apposition, attachment, and adhesion of the conceptus trophectoderm to the endometrial luminal epithelium (LE) of the uterus. Within the outer layer of the conceptus termed the chorion, binucleated trophectoderm cells, termed trophoblast giant binucleate cells (BNC), begin to appear as early as Day 14 (Wooding, 1984). The BNC are thought to be derived from the mononuclear trophectoderm cells (MTC) by a process referred to as mitotic polyploidy, which involves consecutive nuclear divisions without cytokinesis (Wooding, 1992). BNC then appear to fuse with uterine LE to form trinucleate fetomaternal hybrid cells (Wooding, 1984). Other BNCs fuse with the trinucleate cells (and likely each other) to form plaques of multinucleated syncytiotrophoblast that have 20 to 25 nuclei. Trophoblast BNCs of the sheep placenta are analogous in many ways to the giant cells of the syncytiotrophoblast of the human placenta (Hoffman and Wooding, 1993). The syncytial plaques and BNC form specialized structures on the placenta termed cotyledons that interdigitate with the endometrial caruncles of the maternal uterus to form a structure termed a placentome (Igwebuike, 2006). Blood flow to the uterus and from the fetus is predominantly routed to the placentomes, which provides hematrophic nutrition from the mother to the fetus. Other functions of BNC and multinucleated syncytia include production and synthesis of proteins and hormones, like placental lactogen, pregnancy associated glycoproteins and progesterone, that are involved in growth of the uterus and mammary gland and other maternal functions (Wooding, 1992).

The presence of enJSRVs in the ovine uterus was serendipitously discovered during differential display and subtractive hybridization PCR analyses of endometrium from normal fertile and infertile uterine gland knockout (UGKO) ewes (Spencer et al., 1999). A large number of the cDNAs cloned by those techniques exhibited high homology to the *gag* and *env* genes of JSRV. In sheep, enJSRVs are abundant in the epithelia lining the different tissues of the female reproductive tract (vagina, cervix, uterus and oviduct) (Palmarini et al., 2000b; Dunlap et al., 2005). In the uterus, both enJSRVs RNA and protein are detected specifically in the endometrial LE and in the glandular epithelia (GE) (Spencer et al., 1999;

Palmarini et al., 2000b; Palmarini et al., 2001a). In addition, enJSRVs are present in the trophoblast cells of the placenta in a temporal fashion that is coincident with key events in conceptus elongation and onset of trophoblast giant BNC differentiation (Dunlap et al., 2005). Within the placenta, enJSRVs are most abundant in the trophoblast giant BNC and multinucleated plaques of syncytiotrophoblast within the placentomes throughout pregnancy. The RNA of enJSRVs is first detected in the conceptus on Day 12 (Dunlap et al., 2005). Interestingly, HYAL2, a cellular receptor for both JSRV and enJSRVs Env (Rai et al., 2001; Arnaud et al., 2007b), is detected exclusively in the BNC and the multinucleated syncytial plaques of the placenta (Dunlap et al., 2005). These observations led to the hypothesis that enJSRVs and HYAL2 are important for placental growth and differentiation in sheep (Spencer et al., 2007). Indeed, injection of morpholinos that inhibit enJSRV Env production into the uteri of pregnant sheep on Day 8 of pregnancy compromised conceptus elongation, resulting in reduced mononuclear trophoblast cell outgrowth and loss of trophoblast giant BNC differentiation (Dunlap et al., 2006). The biological role of HYAL2 in sheep conceptus development and differentiation has not been determined. Figure 4 presents a current hypothesis on the biological roles of enJSRVs Env and HYAL2 in trophoblast development and differentiation in the sheep conceptus during early pregnancy.

Interestingly, the enJSRVs Env have a high degree of similarity with the oncogenic exogenous JSRV Env; thus, it is tempting to speculate that both endogenous and exogenous JSRV Env share similar mechanisms to induce trophoblast proliferation/differentiation and cell transformation, respectively, since placental morphogenesis has features similar to tumorigenesis and metastasis (Soundararajan and Rao, 2004; Ferretti et al., 2007). Although many of these parallels come from comparisons made with the human placenta, trophoblast cells in general have a high proliferation rate, are migratory and invasive, and have the capacity to evade the immune system, which are also characteristics of cancer cells. Thus, it is likely that enJSRV and JSRV Env mediate their effects through the activation of similar albeit not identical pathways (Pollheimer and Knofler, 2005). Indeed, the Ras-MEK-MAPK, Rac1 and PI3K-Akt-mTOR signalling pathways involved in JSRV-induced cell transformation are important regulators of trophoblast growth and differentiation in human and rodent placentae (Pollheimer and Knofler, 2005).

6. ERVs in cattle and other species

Recent evidence indicates that ERV *env* genes are also expressed in the placenta of cattle (Baba et al., 2011; Koshi et al., 2011). One such element, bERVE-A, has a similar sequence to human syncytin-1 and is expressed specifically in trophoblast BNC, but it lacks an intact *env* sequence (Koshi et al., 2011). Two other proviruses (BERV-K1 and -K2) contain full-length *env* genes and are expressed in the placenta and cultured trophoblast cells of cattle (Baba et al., 2011). These ERVs may have a biological role in development of the placenta, similar to enJSRVs in sheep.

The discovery and characterization of sheep enJSRVs was based on molecular cloning and first generation sequencing technologies because the sheep genome was not sequenced. In contrast, the ERVs expressed in the placentae of Muridae (syncytin-A and -B in mouse, rat, gerbil, vole, and hamster) (Dupressoir et al., 2005a), rabbits (syncytin-Ory1) (Heidmann et al., 2009), and guinea pigs (Vernochet et al., 2011) were discovered using systematic *in silico* searches of sequenced genomes. This approach is expected to increase knowledge of ERVs as the sequence of more mammalian genomes and transcriptomes becomes available through the widespread use of next generation sequencing technologies.

7. Conclusions

ERVs are present in the genomes of all vertebrates (Gifford and Tristem, 2003) and can be used as DNA fossils to unravel virus-host coevolution over millions of years (Coffin, 2004; Chessa et al., 2009). The domestic sheep constitutes a powerful model to study the biological significance of ERVs given the contemporary presence in this animal species of a pathogenic exogenous retrovirus (JSRV) and the biologically active enJSRVs (Arnaud et al., 2007b; Arnaud et al., 2008). During evolution, the first driving force that positively selected and fixed enJSRVs in sheep population was their ability to protect their host against infection by related pathogenic retroviruses (Palmarini et al., 2004; Arnaud et al., 2008). However, the enJSRVs Env are also essential for placental development in sheep (Dunlap et al., 2006). Collective evidence from studies of primates, rodents, and sheep supports the idea that independently acquired, nonorthologous ERVs were positively selected for a convergent physiological role in evolution and development of the placenta. Studies with enJSRVs and JSRV as well as other ERVs expressed in the placenta help us understand how ERVs evolved from infectious elements to essential genes (de Parseval and Heidmann, 2005). The rapid progress in sequencing mammalian genomes and transcriptomes using advanced generation sequencing technologies (Metzker, 2010) is fully expected to considerably expand our knowledge of the biological role of ERVs and other transposable elements in mammals.

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Fig. 1. Life cycle of retroviruses

The retroviral life cycle is arbitrarily divided into two phases, early and late. The stages in each phase are shown above. Interactions between viral and host cell restriction factors occur at every stage of the viral life cycle. The infecting virus attaches to a specific receptor on the cellular plasma membrane with the SU portion of the viral Env protein leading to fusion and entry. Reverse transcription then generates a double-stranded DNA copy of the RNA genome. The provirus is transported into the nucleus and integrated into chromosomal DNA. Transcription by the cellular machinery generates RNA copies that are then translated in the cytoplasm. Virion proteins and progeny RNA assemble at the cell boundary and the plasma membrane, and progeny virus is released into a mature viral particle.

Fig. 2. Representative enJSRVs proviruses present within the sheep genome

Five enJSRVs display an intact genomic organization typical of replication competent proviruses (top). The “W” present in the Gag protein of the two transdominant proviruses enJS56A1 and enJSRV-20 indicates the R21W substitution. The 5' flanking region of enJSRV-20 contains an *env* gene indicated by a box and a question mark (?). Vertical lines and an asterisk (*) represent stop codons, while hatched boxes indicate deletions. enJSRV-6 harbours a recombined structure with internal sequence in the opposite direction compared to the 5' and 3' LTRs of the provirus. The first methionine (indicated by the letter M) of the *env* gene of enJSRV-6 is present after the usual start codon. Figure reproduced from Arnaud and coworkers (Arnaud et al., 2007).

Fig. 3. Dates and events associating the evolutionary history of enJSRVs and their host
Schematic diagram illustrating key events of the evolutionary history of enJSRVs with estimated dates during the evolution of the domestic sheep and the Caprinae. Figure reproduced from Arnaud and coworkers (Arnaud et al., 2008).

Fig. 4. Hypothesis on the biological role of enJSRVs Env and HYAL2 in trophoblast differentiation in sheep

During pregnancy, trophoblast giant binucleate cells (BNC) begin to differentiate from mononuclear trophoblast cells (MTC) on Day 14. First, MTC begin to express enJSRVs envelope (Env) in the conceptus on Day 12 (Step 1). Second, results from microscopy studies support the idea that binucleated trophoblast cells or trophoblast giant BNC are derived from karyokinesis without cytokinesis (endoreduplication) or mitotic polyploidy (Step 2). Next, the newly formed BNC that are co-expressing enJSRVs *env* and *HYAL2* initially fuse with enJSRVs *env*-expressing endometrial luminal epithelial (LE) cells, forming a trinucleated fetomaternal hybrid cell (Step 3). During this period, the BNC and LE cells express enJSRV *env* RNA, whereas only the BNC express *HYAL2*. In fact, *HYAL2* mRNA is not detectable in uterine cells. By Days 20 to 25, virtually all of the endometrial LE cells are fused with the BNC. Fourth, other newly formed BNC fuse with trinucleate cells to form a multinucleated syncytial plaque (Step 4). During most of gestation, the BNC continue to differentiate from the MTC and then fuse with each other and existing multinucleated syncytia to form multinucleated syncytial plaques with 20 to 25 nuclei. The multinucleated syncytial plaques and BNC form the basis of the cotyledons of the placenta that interdigitate with caruncles of the endometrium to develop and form placentomes.