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Paleovirology of 'syncytins', retroviral env genes exapted for a role in placentation

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Review

The development of the emerging field of 'paleovirology' allows biologists to reconstruct the evolutionary history of fossil endogenous retroviral sequences integrated within the genome of living organisms and has led to the retrieval of conserved, ancient retroviral genes 'exapted' by ancestral hosts to fulfil essential physiological roles, syncytin genes being undoubtedly among the most remarkable examples of such a phenomenon. Indeed, syncytins are 'new' genes encoding proteins derived from the envelope protein of endogenous retroviral elements that have been captured and domesticated on multiple occasions and independently in diverse mammalian species, through a process of convergent evolution. Knockout of syncytin genes in mice provided evidence for their absolute requirement for placenta development and embryo survival, via formation by cell–cell fusion of syncytial cell layers at the fetal–maternal interface. These genes of exogenous origin, acquired 'by chance' and yet still 'necessary' to carry out a basic function in placental mammals, may have been pivotal in the emergence of mammalian ancestors with a placenta from egg-laying animals via the capture of a founding retroviral env gene, subsequently replaced in the diverse mammalian lineages by new env-derived syncytin genes, each providing its host with a positive selective advantage.

1. Introduction

Much to the surprise of biologists, the determination of genomic sequences from eukaryotes revealed that, in the course of evolution, the DNA of almost all eukaryotic organisms had been colonized by countless mobile genetic elements [1,2]. Mobile elements, also known as transposable elements (TEs), can both multiply copies of themselves and integrate these copies into the genome of their host's germline, become 'fixed' and be transmitted vertically to the offspring in a Mendelian fashion. They could be identified at the nucleotide sequence level as repeated sequences with specific features and/or by homology with extant, active mobile elements. Similar to paleontological fossil records that are used to reconstitute the evolutionary history of species, more or less well-conserved traces of these elements could be retrieved, which indicated that they had been invading eukaryotic genomes over millions of years and had coevolved with them.

Two main classes of TEs can be distinguished: class I that includes long terminal repeat (LTR) retrotransposons, non-LTR retrotransposons and endogenous retroviruses (ERVs), and class II that comprises DNA transposons [3]. Whereas DNA transposons propagate via an excision and reintegration mechanism using a transposase enzyme they encode, autonomous retrotransposons and ERVs have an RNA genome and code for the enzymes allowing them to make a cDNA copy of their genomic RNA (reverse transcriptase) and integrate it into the genome of their host (endonuclease or integrase). Non-autonomous retrotransposons are trans-mobilized by the replicative machinery of active autonomous retrotransposons. Recently, both DNA viruses and RNA viruses (other than

retroviruses) were also found to have left within eukaryotic genomes integrated DNA copies, designated as endogenous viral elements (EVEs; [4,5]), albeit at a frequency much lower than that of TEs. EVEs are thought to result either from direct insertion by host recombination mechanisms such as non-homologous end joining in the case of DNA viruses or from transposition through the use of the enzymatic machinery of retrotransposons in the case of RNA viruses [4]. Altogether, these elements occupy a substantial fraction of many eukaryotic genomes. For instance, they make up at least 45% and 40% of the human and mouse genomes, respectively [6,7]. Most of the TE sequences have degenerated and have been extensively rearranged in evolution compared with the ancestral inserted elements, but the youngest are still intact and active in a number of host species [8].

Transposons are potentially pathogenic elements that can be gene-disruptive depending on their integration sites and all the more so when amplification bursts occurred, as can be traced through evolution by the accumulation of multiple copies that inserted during the same time interval. But host organisms have developed multiple defence mechanisms to counteract and restrict TE invasions. In turn, TEs are subject to high mutation rates that allow them to escape host restriction factors, which consequently require new restriction mechanisms to be selected for by the host. The outcome of this struggle for survival on both sides turns out to be a motor for evolution [9–11].

Although endogenous mobile elements can be considered as mere 'genetic parasites', generator of the so-called 'junk' DNA, there is accumulating evidence that some of them were repeatedly co-opted by their host genomes to play beneficial functional roles [12–14]. Actually, the fact that such an extensive fraction of many eukaryotic genomes is composed of TE sequences should have led to the inference that their presence could be accommodated and was not irremediably detrimental to the fitness of the organisms harbouring them. In several cases, they even appear to have provided ultimately a selective advantage. The effects of endogenous TEs encompass genome remodelling owing to DNA recombination and unequal crossover, changes in gene expression through transcription regulation and splicing variations, and even contribution to protein-coding sequences [15–18]. In the context of the latter, the present review will focus on the ERV class of TEs and address the process of endogenization of retroviruses, the co-optation of protein-coding genes from ERVs in diverse mammalian species and more specifically the diversion of retroviral gene functions to perform specialized tasks in the placenta.

2. Retrovirus endogenization

As mentioned above, retroviruses replicate via an RNA intermediate [19]. The viral genomic RNA present in the infectious particles serves as a template for the synthesis of a cDNA copy which is then integrated into the genome of the target cell in the form of a 'provirus'. Repeated transcription of the provirus allows amplification of viral genomes and production of new infectious virus particles. Infection of the germline, integration and fixation of proviruses at defined positions in the host genome and vertical Mendelian transmission to the offspring constitute the successive stages of the process of endogenization (figure 1). The resulting

ERVs that have conserved a recognizable full-length structure display the typical genetic organization of proviruses from known infectious retroviruses. Most of them harbour only the three genes contained in the genome of simple retroviruses, *gag*, *pol* and *env*, flanked by two LTRs. The *gag* gene encodes a precursor of the subunits that serve as components of the viral capsid, the *pol* gene a polyprotein that is processed into the constituents of the viral enzymatic machinery required to convert the viral RNA into the double-stranded proviral DNA form, including reverse transcriptase, RNase H and integrase, and the *env* gene, a glycoprotein which is inserted into the virion membranous envelope and forms spikes at the surface of the retrovirus particles.

During evolution, successive bursts of amplification within host germlines have led to the formation of ERV repeat families that occupy a significant fraction of mammalian genomes (e.g. 8% and 10% in humans and mice, respectively) [19,20]. Over time, almost all ERVs not subject to any selective pressure have been mutated to various degrees, including large deletions, insertions and extensive sequence rearrangements. Most have been left as scattered and truncated fragments. Very often, solitary LTRs have been generated by homologous recombination between the two LTRs from single proviruses and loss of the internal sequences.

However, in some mammalian species, fully functional proviruses persist. Such 'young' ERVs can be reactivated to produce infectious virions that are transmitted horizontally and can even reinfect the germline to form proviruses that can become fixed as new ERVs at novel positions in the host genome. This is notably the case for the mouse leukemia viruses (MLVs) and the mouse mammary tumor viruses [21], the endogenous Jaagsiekte sheep retroviruses (enJSRVs) [22] and the more recently identified koala retrovirus (KoRV) [23]. The last is spreading among koala populations where it may lead to leukaemia and lymphoma, although some preserved, isolated populations are still virus-free. KoRV, present in affected animals as both an infectious exogenous retrovirus and an endogenized ERV within the koala genome, is being considered as an attractive model to study the process of endogenization in the evolution of a species.

In other rare situations, single genes from endogenized ERVs have been conserved over millions of years of evolution, whereas the rest of the proviral sequences have degenerated or been lost (figure 1). Maintenance of a functional gene of retroviral origin in a group of species belonging to the same clade strongly suggests that such a gene provides a selective advantage to its hosts. Remarkably, retroviral *env* genes were found to have been repeatedly 'captured' and 'domesticated' to fulfil critical physiological functions in the placenta of several mammalian species belonging to separate clades.

3. The retroviral envelope glycoprotein

The envelope glycoprotein (Env) of enveloped viruses is essential for viral entry by recognizing susceptible cells and inducing fusion of the virion envelope with the cell plasma membrane, allowing the release of the nucleocapsid into the cell cytoplasm [24]. The *env* gene of retroviruses encodes a precursor protein which is cleaved, during transport through the host cell secretory pathway, by a cellular furin protease to generate two fragments, the surface (SU) and the transmembrane (TM) subunits (figure 2a) [25]. Both subunits remain associated
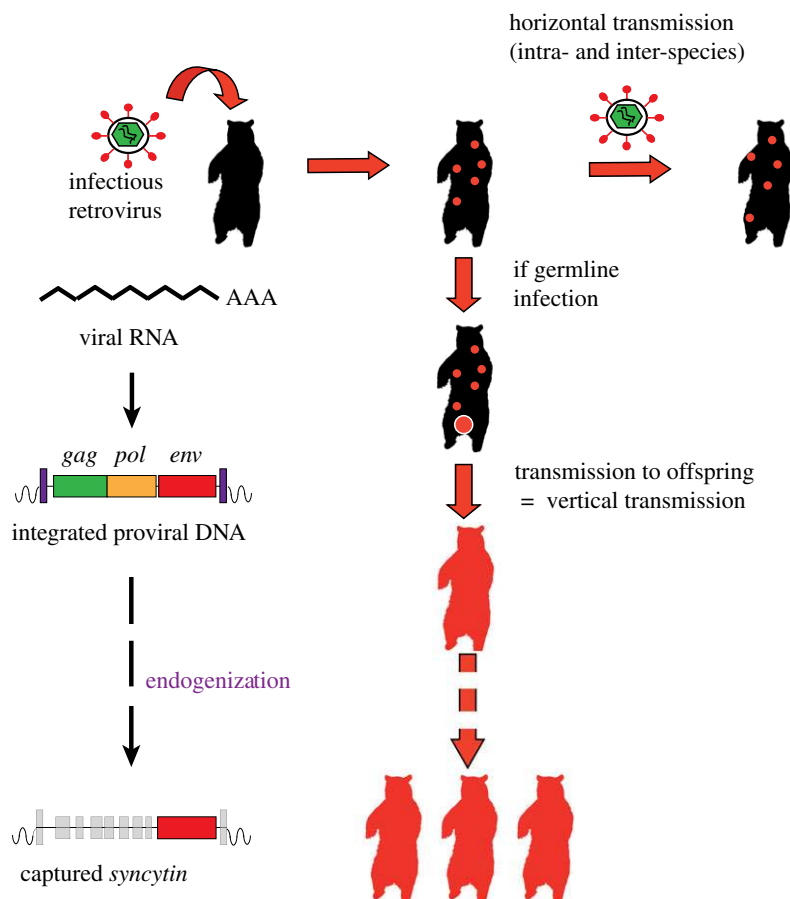
Figure 1. Retrovirus endogenization and *syncytin* gene capture in mammalian species. Retroviruses are able to reverse transcribe their genomic RNA into a cDNA copy that integrates into the genome of infected cells as a 'provirus' which contains the three viral genes *gag*, *pol* and *env* (left). Retroviral infection leads to proviral insertion into a limited number of cells of the infected animal. Production of new infectious virions results in horizontal transmission of the virus (top right). In occasional cases where a retrovirus infects germline cells, the integrated proviral DNA is transmitted vertically to the offspring (centre). The retrovirus has been 'endogenized' and is now present in all the somatic and germline cells of the animal. During evolution, most of the endogenous retrovirus-derived genes are disrupted by multiple mutational events but occasionally one of them, such as the *env* gene exemplified here (bottom left), may be preserved and remains functional over several million years, playing a role in the physiology of its host.
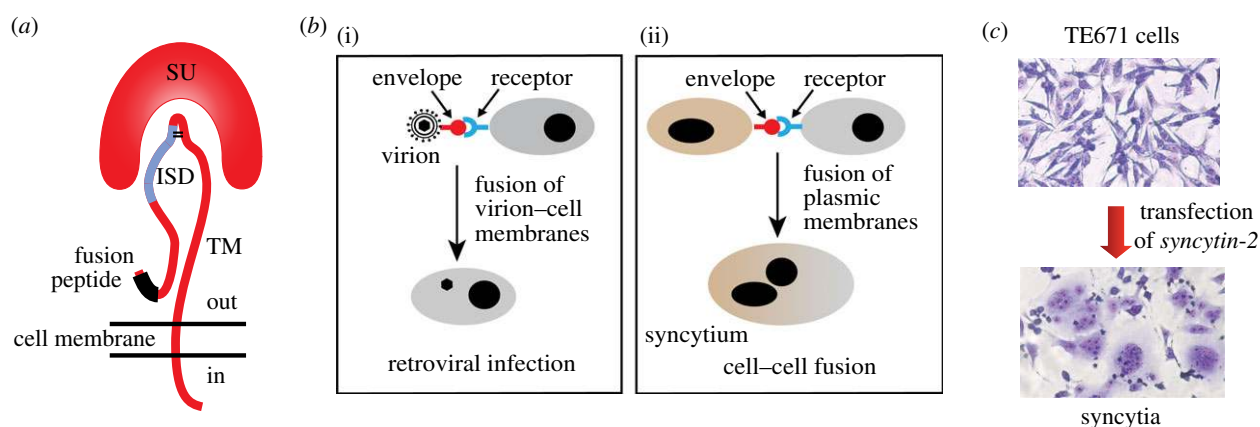

Figure 2. The retroviral envelope glycoprotein. (*a*) Structure of the retroviral envelope protein with the SU and TM subunits, the fusion peptide and the immunosuppressive domain (ISD). (*b*) Consequences of the interaction between a retroviral envelope protein and its cognate receptor: virion–cell membrane fusion and virus entry into the target cell (i) or cell–cell membrane fusion and formation of a syncytium (ii). (*c*) Cell–cell fusion and formation of multinucleated syncytia induced by transfecting human TE671 cells with a *syncytin-2* expression vector (May–Grünwald–Giemsa staining).

as heterodimers which are further assembled into Env trimers at the surface of the virion envelope membrane. The SU subunit displays a domain which specifically interacts with a receptor on the surface of cells susceptible to the virus, whereas the TM subunit triggers the fusion of the virus and cell membranes by insertion of its N-terminal fusion peptide into the membrane of target cells (figure 2a,b). The TM subunit also contains a transmembrane domain anchoring the Env heterodimer into the virion envelope and a highly conserved immunosuppressive domain (ISD).

The immunosuppressive activity of retroviral Env proteins had been first reported in *ex vivo* studies [26] and was

further substantiated in our laboratory using *in vivo* tumour rejection assays that measured the ability of the proteins, when expressed in allogeneic tumour cells normally rejected by engrafted mice, to allow these cells to escape, at least transiently, immune rejection [27]. This approach led to the delineation of the ISD within a 20 amino acid-long peptide and to the identification of two amino acid positions where introduction of specific mutations could abolish the immunosuppressive activity without affecting the fusogenicity and infectivity of the protein [28]. The ISD was next demonstrated to be critical for the propagation of the retrovirus itself [29]: the presence of the ISD-inactivating mutations within the *env* gene fully impaired the replication of Friend MLV in normal immunocompetent mice, whereas the mutant virus replicated with the same efficiency as the wild-type, both *ex vivo* in cultured cells and *in vivo* in irradiated immunocompromized mice. Finally, specific cell depletion experiments carried out using the above virus–host murine model demonstrated that the identified ISD had a pleiotropic effect, with impairment of both the innate and adaptive immune responses. Thus, retroviral Envs are more complex than initially suspected, being multifunctional proteins that can act both as mechanical effectors for membrane fusion, and as immunological effectors required for repressing the anti-viral host immune response.

Interestingly, the Env glycoproteins encoded by several endogenized ERV *env* genes have kept some of the properties initially displayed by the Env of their ancestral infectious retrovirus, such as recognition of a specific cell receptor, fusogenicity and/or immunosuppressive activity. Conservation of such features may indicate a significant physiological role in the host cells where the genes are expressed. In the mouse, for instance, the ERV *env*-derived genes, *Fv4* and *Rcmf*, were shown to be involved in a process of receptor interference, conferring to the cells where they are expressed resistance to superinfection with exogenous retroviruses by downregulating their receptor on the target cell surface [30]. The same phenomenon was found to occur in the case of the enJSRV *env* genes [22]. In the case of several enveloped viruses, the Env protein synthesized by infected cells and exposed at their surface can confer on them the ability to fuse with neighbouring uninfected cells that express the appropriate receptor, resulting in the formation of multinucleated giant cells called syncytia (figure 2*b*) [31]. In this context, several placenta-specific genes encoding fusogenic Env proteins co-opted from members of distinct ERV families have been identified within the genome of various mammalian species.

4. Human endogenous retrovirus *env* genes

Comprehensive screening for identifying human ERV (HERV) *env* genes with long open reading frames (ORFs) within the human genome disclosed 18 genes encoding a putative envelope protein [32,33]. Among those potentially functional, the first to be described was *ERV3*, a proviral gene specifically expressed in the placenta and conserved in the Hominoid and Old World monkey families [34]. The ERV3 protein lacks a hydrophobic transmembrane anchoring domain and therefore the ability to operate as a fusogenic protein, and was suggested to be involved in the proliferation and/or differentiation of the trophoblast. However, the absence of the *ERV3* gene in the gorilla [34] and the fact

that a premature stop codon was found to be present in a homozygous state in 1% of the human population ruled out that this otherwise highly conserved gene might be critical for reproduction or survival [35]. However, one could envision that the human *ERV3* gene has played a role in the past, but may now be undergoing a process of progressive loss of function with compensatory replacement by another gene(s).

Next, attention was focused on two envelope glycoproteins, one encoded by a member of the HERV-W family [36,37] and the other by a member of the HERV-FRD family [38]. Both *env* genes display placenta-specific expression and induce syncytium formation when introduced into cultured cells (figure 2*c*), which led to their designation as *syncytin-1* and *syncytin-2*, respectively. Each protein binds a distinct receptor: *syncytin-1* interacts with the neutral amino acid transporter ASCT2 [36] and *syncytin-2* with the multipass transmembrane protein MFSD2 [39]. Comparative analysis of the syntenic regions within the genomes from various species revealed that the *syncytin-1* gene entered the genome of primates before the separation of Hominoids from Old World monkeys, being conserved only in Hominoids [40], whereas *syncytin-2* was found to be conserved in the genome of all primates, except prosimians [38].

Determination of the presence or absence of each *syncytin* gene in various extant species provides a paleovirological record of its evolutionary history which, together with the otherwise determined phylogeny of the present-day species, allows the dating of the insertion of its parental ERV into the genome of a common ancestor. Thus, the age of the *syncytin-1* gene could be estimated to be around 30 million years (Myr) and that of *syncytin-2* to be older, at more than 45 Myr. Compellingly, for both genes, the conservation of an intact ORF in multiple related species, with a low ratio of non-synonymous versus synonymous mutation rates ($dN/dS < 1$) indicative of a 'purifying selection' acting on the genes, and the very low levels of polymorphism within the human population argue for an essential physiological role [41]. Several features of both human syncytin proteins indicated that they are instrumental in the formation of a major component of the placenta, i.e. the syncytiotrophoblast.

5. *Syncytins* in the human placenta

The placenta is a transient autonomous organ of embryonic origin whose main function is to mediate mutual metabolic exchanges between fetus and mother. It also provides an environment of immune tolerance ensuring the protection of the 'fetal allograft', which expresses 'foreign' paternal antigens, within the mother's body. The placental tissue, or trophoblast, is formed by peripheral cells of the blastocyst, the cytotrophoblasts, which attach to the uterine wall. Cytotrophoblasts are among the rare cells of the human body, together with the myoblasts, the macrophages and the gametes, able to undergo cell–cell fusion. Beginning at embryonic day 6 (E6), mononucleated cytotrophoblasts fuse together into the syncytiotrophoblast, a highly invasive multinucleated syncytial layer that mediates implantation of the embryo into the maternal endometrium [42]. From E15, the basic functional unit of the placenta composed of the chorionic villi, starts to develop. The villi either float freely in maternal blood spaces or are anchored into the uterine wall (figure 3*a*).
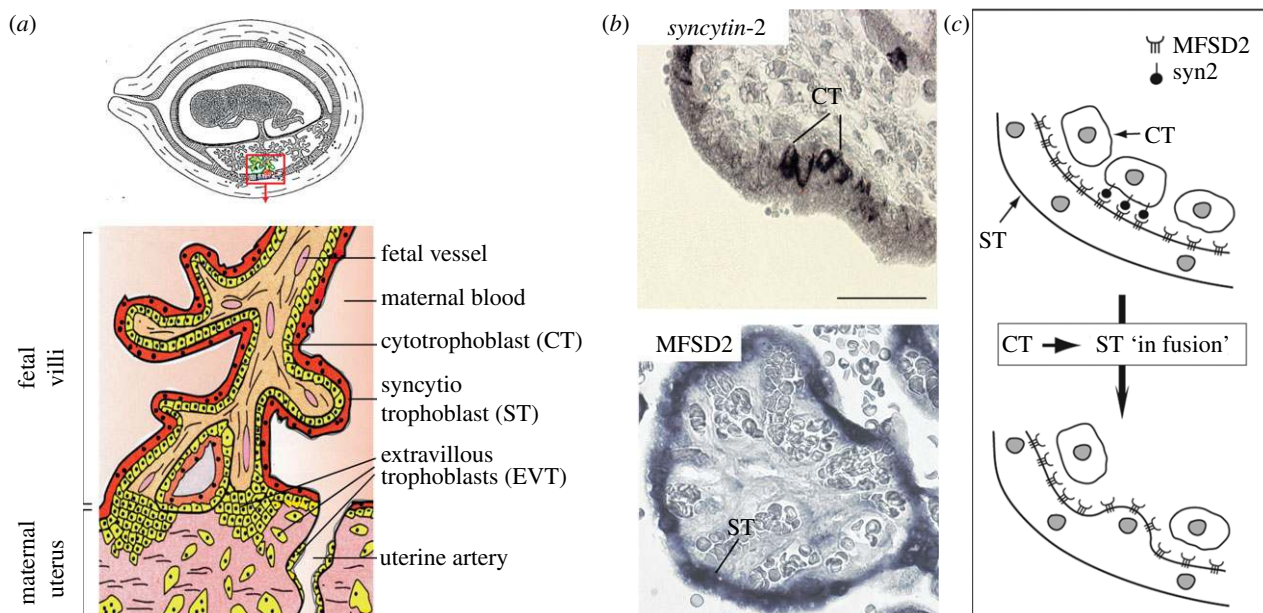
Figure 3. Involvement of *syncytin-2* and its cognate receptor MFSD2 in human placentation. (*a*) Schematic of a human feto–placental unit and (enlarged) of a placental villus. (*b*) *In situ* analysis of human placental villi sections for *syncytin-2* and MFSD2 expression. MFSD2 expression is detected exclusively within the syncytiotrophoblast layer (ST), whereas *syncytin-2* expression is restricted to underlying mononucleated cytotrophoblasts (CT). (*c*) 'In fusion' model for syncytio-trophoblast formation, where interaction between *syncytin-2* and MFSD2 results in polarized fusion of the cytotrophoblasts into the syncytiotrophoblast. Adapted from Esnault *et al*. [39].

At this stage, fetal blood vessels are embedded in the internal mesenchymal stroma, whereas the syncytiotrophoblast covers the outside surface of the villi, in direct contact with maternal blood. The syncytiotrophoblast regenerates and expands by fusion of underlying cytotrophoblasts. This essential tissue plays multiple roles, including exchange functions between mother and fetus, hormone secretions, regulation of the immune response and protection against pathogens [43]. Finally, extravillous cytotrophoblasts originating from an independent differentiation pathway and located at the base of anchoring villi gain an invasive phenotype, migrate into the maternal decidua and later differentiate into giant cells in the myometrium. They also invade the uterine 'spiral arteries' where they replace the endothelial cells and remodel them into dilated vessels, thus increasing the maternal blood flow to the fetus (figure 3*a*) [44].

In situ analysis of *syncytin* expression suggested that both genes were involved in placental development. *Syncytin-1* was found to be expressed throughout the placenta in all the trophoblast cell lineages [45,46]. Interestingly, the placental transcription factor GCM1 was shown to control *syncytin-1* expression and to display an expression profile coinciding with that of *syncytin-1* in placental tissues [47]. However, no clear results have yet been reported with regard to the localization of the expression sites for ASCT-2, the syncytin-1 receptor, in trophoblast cells, which seems to depend on the antibodies used, so that evidence for syncytin-1 involvement in syncytiotrophoblast formation is not fully conclusive at this time. By contrast, *syncytin-2* expression takes place in only a fraction of the villous cytotrophoblasts [48], whereas expression of the gene for its receptor, *MFSD2*, is detected in the syncytiotrophoblast (figure 3*b*) [39]. Coordinated expression of *syncytin-2* and *MFSD2* in distinct cell types may drive the incorporation by fusion of the mononucleated cytotrophoblasts into the adjacent syncytiotrophoblast, while impairing fusion of the cytotrophoblasts between themselves.

Such a process of polarized 'in-fusion' may promote the growth and renewal of the syncytiotrophoblast during pregnancy (figure 3*b*) [39]. In primary cultures of spontaneously differentiating cytotrophoblasts, silencing of *syncytin-2* and, to a lesser extent of *syncytin-1*, either by the addition of antisense oligonucleotides or by RNA interference with small inhibitory RNAs, greatly interferes with cell–cell fusion [49,50]. Altogether, these data provide evidence for a major role of *syncytin-2* (likely in concert with *syncytin-1*) in the differentiation of the syncytiotrophoblast. Although additional cellular factors have been previously reported to contribute to the fusion process (e.g. phosphatidylserine externalization, the genes encoding connexin 43, cadherin 11 and CD 98, the *zona occludens-1* (*ZO-1*) gene) [51,52], the real effectors are most probably the *syncytin* genes, owing to their intrinsic fusogenicity. It has to be stressed that it has not been possible to substantiate a role for syncytins in syncytiotrophoblast formation at early implantation (i.e. at approx. E6), owing to the limited availability of placental material at such an early stage in humans.

6. Mouse *syncytins*: a genetic clue for a role in placentation

With the purpose of developing an animal model to investigate the role of syncytin proteins in placental development, the mouse genome was screened for syncytin-encoding genes. Remarkably, bioinformatic analysis of the fully sequenced mouse genome led to the discovery of two genes encoding retroviral Env proteins, designated *syncytin-A* and *-B*, distinct from the human *syncytin-1* and *-2* genes, but sharing the same characteristics [53]: they display placenta-specific expression, are fusogenic, and have been highly conserved since their integration into the genome of a muroid ancestor more than 25 Myr ago (Ma).
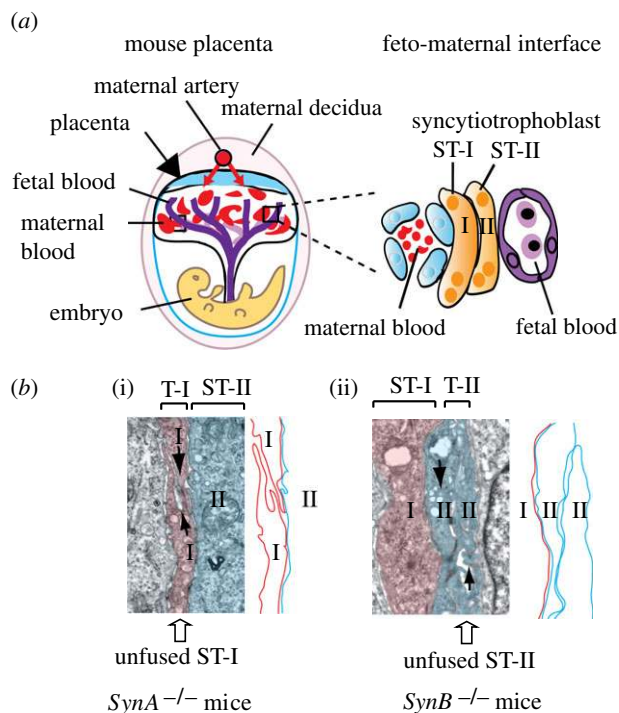
Figure 4. Both murine *syncytin* genes are required for the formation of the murine syncytiotrophoblast double layer. (*a*) Scheme of the mouse placenta with an enlarged representation of the feto-maternal interface highlighting the presence of two syncytiotrophoblast layers (ST-I and ST-II). (*b*) Electron microscopy analysis of the placental feto-maternal interface of mice deficient for *syncytin-A* (i) or *syncytin-B* (ii), showing fusion defects of syncytiotrophoblast layers ST-I and ST-II, respectively. Adapted from Dupressoir *et al.* [54,55].

In the mouse placenta, the fetal capillaries are separated from the maternal blood lacunae by two distinct layers of syncytiotrophoblast (ST-I and ST-II; figure 4). Although the gross architecture of the mouse and human placentae differ, a functional analogy can be made between the single human syncytiotrophoblast layer and the murine ST-I and ST-II layers: the two murine layers could be considered as a unique structure because they are in intimate contact, with gap junctions establishing continuity between their respective cytoplasm (for general reviews comparing the structure and function of the mouse and human placentae, see Georgiades *et al.* [56] and Watson & Cross [57]). The mouse *syncytin-A* gene was found to be expressed in the ST-I layer, proximal to maternal blood spaces, whereas *syncytin-B* expression was detected in the ST-II layer, close to fetal blood vessels [58]. As was the case for human *syncytin-1*, *syncytin-B* expression is controlled by the Gcm1 transcription factor, in agreement with the fact that the *syncytin-B* and *Gcm1* genes are both expressed in the ST-II layer. Knocking out the *syncytin-A* gene in mice had dramatic effects, since homozygous mutant embryos died at mid-gestation [54], displaying disruption of the early placental architecture, accumulation of unfused cytotrophoblasts and defective cell–cell fusion within the ST-I layer (figure 4). By contrast, invalidation of the *syncytin-B* gene had only mild effects, since *syncytin-B* null embryos were viable, showing only limited late-onset growth retardation and a slight decrease in the number of neonates [55]. Yet, the *syncytin-B* null placenta displayed an altered ST-II layer with unfused, apposed cytotrophoblasts and enlarged maternal blood spaces (figure 4).

Analysis of the *syncytin-B* null mutant transcriptome, compared with that of wild-type placenta, showed induction of the gene encoding connexin-30, a gap junction protein, which was found to accumulate at the maternal–fetal interface within the area of the defective unfused ST-II cell layer. These results suggested that compensatory mechanisms mediated by gap junctions, which promote direct intercellular communications, might counteract the fusion defects and allow embryos lacking the *syncytin-B* gene to survive. Remarkably, double knockout embryos lacking both *syncytin-A* and *syncytin-B* displayed an aggravated phenotype as compared with the *syncytin-A* single knockout embryos, since they died earlier [55]. Thus, both the ST-I and ST-II syncytial layers cooperate to preserve the structural and functional integrity of the maternal–fetal interface. These mutant mice provided the first demonstration of the critical role played by *syncytin* genes in placentation. They also constitute a model to investigate further the potential involvement of *syncytin* gene mutations in placental diseases associated with cell–cell fusion defects.

7. *Syncytins* are widespread in eutherian mammals

Although they share similar features, the primate and muroid *syncytins* are clearly not orthologous genes—they are not syntenic in the two species clades—indicating that they are the result of independent gene captures that occurred separately in the genome from ancestors of each lineage. Furthermore, a fifth *syncytin* gene, *syncytin-Ory1*, distinct from each of those just listed, has been identified in yet another mammalian lineage, the Leporidae family (rabbit and hare) [59]. The *syncytin-Ory1* gene codes for a placenta-specific envelope protein endowed with fusogenic activity and has been conserved for over 12 Myr. Its receptor, ASCT-2, is the same as that of the human syncytin-1 protein. *Syncytin-Ory1* expression was detected in the placenta junctional zone, where the invading placental syncytia come into contact with the maternal decidua, suggesting its possible involvement in syncytiotrophoblast formation. More recently, a sixth functional *syncytin* gene, *syncytin-Car1*, was found to be present in the 26 species of carnivores investigated [60]. Carnivores are members of the Laurasiatheria superorder, which diverged from the Euarchontoglires more than 80 Ma. This makes *syncytin-Car1* the oldest known *syncytin* gene. Both the dog and cat *syncytin-Car1* display fusogenic activity and are specifically expressed in the placenta within the syncytiotrophoblast layer at the maternal–fetal interface [60].

Altogether, these studies provide clear evidence that a number of retroviral *env* genes have been independently co-opted on multiple occasions in the course of mammalian evolution (figure 5) and may have thus repeatedly prompted the emergence of a syncytial maternal–fetal interface, exemplifying a remarkable phenomenon of evolutionary convergence. Interestingly, comparative anatomy reveals that the placenta is the most variable organ among mammalian species (reviewed in Wooding & Burton [62]; figure 5). Placentae essentially differ in the extent of uterine tissue invasion by the trophoblast cells covering the blastocyst at implantation and by the architecture of the resulting maternal–fetal interface. In the epitheliochorial placenta (horse and pig), the fetal trophoblast is composed of unfused, mononucleated
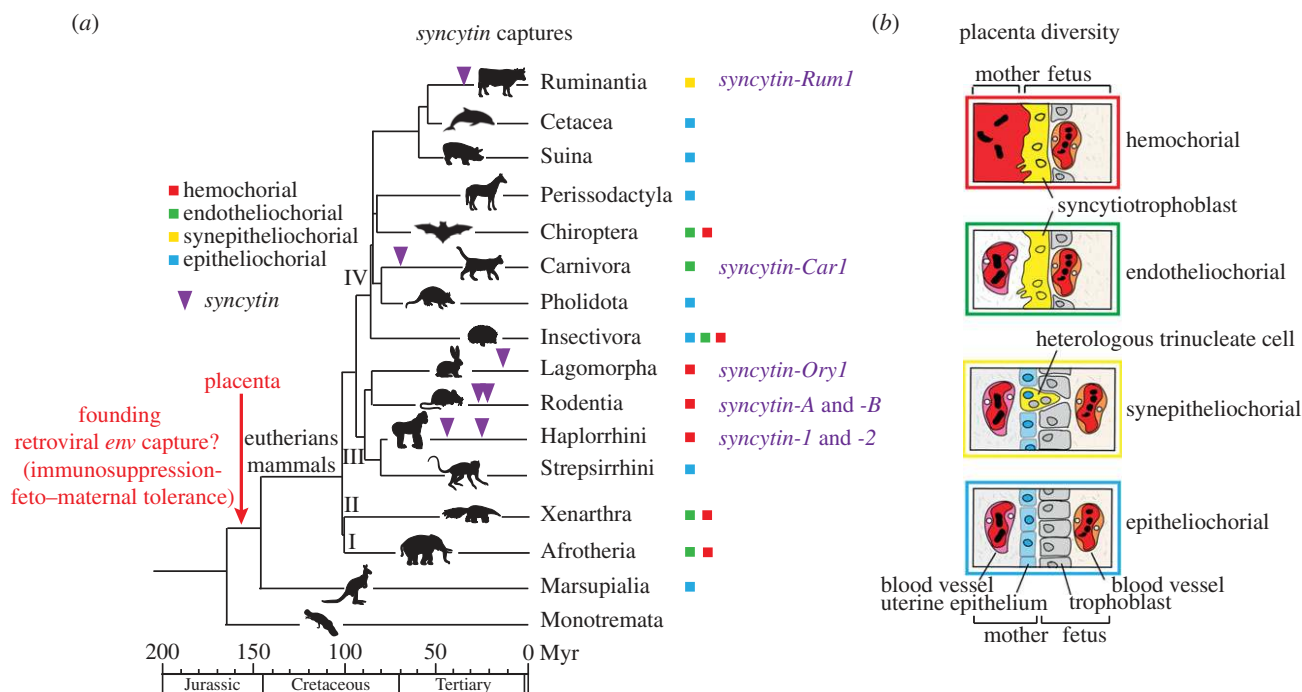
Figure 5. Multiple *syncytin* gene captures and diversity of placental structures in eutherian mammals. (*a*) Phylogenetic tree of mammals, with the four major clades of eutherians: (I) Afrotheria, (II) Xenarthra, (III) Euarchontoglires and (IV) Laurasiatheria (adapted from Meredith *et al.* [61]). The four basic types of placentation are indicated by coloured squares (the colour code corresponds to that of the boxes that frame the images shown to the right). The time of insertion of the different *syncytin* genes identified to date is indicated. Branch length is proportional to time (in million years, Myr). (*b*) Schematic colour-coded representation of the maternal–fetal interface in the four main types of placental structures. Placental types are classified from top to bottom in the order of decreasing extent of syncytialization and invasive properties.

cytotrophoblasts that form a monolayer adjacent to the uterine epithelium. In the three other types of placental interface, the synepitheliochorial (e.g. ruminants), the endotheliochorial (e.g. carnivores) and the hemochorial (e.g. humans, mice and rabbits) types, the degree of invasiveness of the trophoblast increases from one type to the next, respectively, and is accompanied by sequential loss of the intervening maternal cell layers (epithelial and endothelial cells), up to direct contact between the syncytiotrophoblast and maternal blood in the case of the hemochorial placenta (figure 5). However, the various placental structures cannot be predicted merely based on species taxonomy, and their evolutionary pathways are still a matter of debate [63]. Along this line, it seems relevant to ask in what measure, depending on expression pattern, intrinsic fusogenic activity or receptor identity and localization, the *syncytin* genes present in each species may account for the observed variability in the morphology and organization of the utero–placental interface.

In this context, it is of interest to note that a seventh *syncytin* gene was recently discovered in the suborder Ruminantia [64], in whose species the placenta lacks an extended syncytium layer, but displays synepitheliochorial placentation, which involves a very limited cell fusion process, with only trinucleate cells (or small multinucleated syncytial plaques in the case of sheep) being formed and with evidence for heterologous fusion between cells of fetal and maternal origin, a feature not found among the other eutherian mammals. This gene, named *syncytin-Rum1*, was first identified in the cow, where it was shown to be specifically expressed in the placenta. Next, it was found to be also present within the genome of sheep, as well as of 14 other higher ruminant species that were investigated, such as cervids, giraffes and antelopes, indicating that it has been conserved for

more than 30 Myr. Both the cow and sheep orthologues were found to display fusogenic activity in *ex vivo* assays by conferring infectivity on 'pseudotypes' (i.e. defective retroviral particles) and triggering cell–cell fusion. *In situ* hybridization on placenta sections revealed specific expression of *syncytin-Rum1* in the trophoblast binucleate cells, consistent with a role in the formation, by heterologous cell fusion with uterine cells, of the trinucleate cells of the cow and the syncytial plaques of the ewe. *Syncytin-Rum1* is common to all ruminant species displaying a synepitheliochorial placenta but is not found in any other mammalian species. Therefore, its capture may have contributed to the emergence of this unique placental structure. Altogether, the available data strongly support the notion that the exaptation of *syncytin* genes has driven major morphological innovations in mammalian placentae, and provides new molecular clues to understand the remarkable transformations that took place during placenta evolution.

Finally, it has to be noted that the involvement of *env* gene capture in placenta development may not be restricted to the bona fide *syncytin* genes endowed with fusogenic activity. For instance, in sheep, injection of antisense morpholino-oligonucleotides *in utero* showed that the enJSRV *env* genes— acquired through the recent endogenization of an infectious JSRV retrovirus in the Caprinae (sheep/goat) lineage 5–7 Ma (but not found in the cow)—are involved in the control of placental growth and differentiation at early stages of implantation, independently of cell fusion [65]. Another placenta-specific ERV *env* gene, designated *env-Cav1*, was discovered within the genome of the guinea pig [66], which is a member of the Caviomorpha rodent suborder, distant from muroids. An orthologous gene with a conserved ORF was found to be present in all the Caviomorpha species that were investigated, consistent with a time of insertion more than 30 Ma. Guinea pig *env-Cav1* is

specifically expressed at the level of the placenta junctional zone, suggesting a role in the invasion of the uterine tissues by cyto-trophoblasts, as hypothesized for *syncytin-1* in humans. No fusogenic activity of the *env-Cav1*-encoded protein could be detected, indicating that this gene may not play a role in placental architecture, as that played by the previously identified classical syncytins, but be involved in invasiveness and/or exert immunosuppressive functions (see §8).

8. Conclusion: a model for retroviral *syncytin* capture, life and death in the natural history of placental mammals

The determination of an ever increasing number of whole genome sequences from living organisms allows us to reconstruct the evolutionary history of fossil retroviral sequences, some of which have conserved an activity beneficial to their host over millions of years. The development of this emerging field of 'functional paleovirology' has led to the retrieval of genes of ancestral retrovirus origin encoding proteins that have been 'exapted' to fulfil essential physiological roles, *syncytin* genes being undoubtedly among the most remarkable examples. Indeed, *syncytins* are 'new' genes encoding proteins derived from the envelope protein of endogenous retroviral elements that have been captured and domesticated on multiple occasions and independently in diverse mammalian species, through a process of convergent evolution. Knockout of *syncytin* genes in genetically modified mice provided evidence for their absolute requirement for placenta development and embryo survival, via formation by cell–cell fusion of syncytial cell layers at the fetal–maternal interface. Thus, viral genes initially indispensable for the completion of the multiplication cycle of retroviruses appear to have been converted to genuine 'cellular genes' endowed with an essential physiological role.

These genes of exogenous origin and acquired 'by chance' raise a paradox, as they are 'necessary' to carry out a basic function common to placental mammals, and yet they appear to have been acquired lately. Indeed, although the capture of some of them dates back to up to 80 Myr, the presently identified *syncytins* can still be considered as relatively recent genes by comparison with the date of emergence of a primitive placenta in a mammalian ancestor (approx. 150 Ma). To resolve this paradox, an evolutionary model can be proposed which is consistent with our present knowledge on the paleontology of retroviral elements, and the functional properties identified for the *env* genes, either from present-day infectious retroviruses or from anciently captured ERVs.

The model states that a pivotal event in the emergence of placental mammals has been the capture of a founding retroviral *env* gene, but that this ancestral *env* gene has been subsequently replaced in the diverse lineages emerging in the course of the mammalian radiation upon successive and independent germline infections by new retroviruses and co-optation of their *env* gene, each new gene providing its host with a positive selective advantage (figure 5). Such a hypothesis would account for the evolutionary transition from egg-laying to placental animals as well as for the diversity in both the nature and time of insertion of the captured *syncytins* that can be currently found. It would also rather simply account for the multiplicity of placental structures

that have emerged, concomitantly with the diversity of the captured *env* genes. A consequence of this model is that evidence should exist for 'lost *syncytins*' in eutherian mammals, and this is precisely what was found in a recent study of another human envelope protein gene, belonging to a HERV-V provirus, named *envV*, which is also specifically expressed within the human placenta, and whose putative role in human placentation remains to be investigated since it was found to be non-fusogenic [67]. Actually, this *env* gene entered the primate genome concomitantly with *syncytin-2*, that is, more than 45 Ma as inferred from its present status in primates. Interestingly, it was recently shown that the gene is fusogenic in the Old World monkeys where it most probably still behaves as a bona fide *syncytin*, whereas its fusogenic activity appears to have been lost in higher primates (including humans) and in some New World monkeys [68]. This situation well illustrates how a captured retroviral gene may undergo distinct evolutionary fates depending on the evolution of the host itself, in a complex reciprocal interplay. Other *env* genes, such as *ERV3*, might also correspond to degenerating *syncytins*, on their way to be replaced by new emerging *syncytins*, in a continuous evolutionary process whereby incoming retroviruses would play the role of 'generators of diversity'.

A question still pending on the primary role of syncytins in the emergence of placental mammals concerns the function of the ancestral *env* gene that was originally co-opted. Indeed, we now know from the data reported above that Env proteins are not only involved in membrane fusion (a property necessary for syncytiotrophoblast formation) but also are bona fide immunosuppressive effectors. As mentioned above, the TM subunit of the Env protein from infectious retroviruses contains an ISD endowed with an immunosuppressive function critical for the propagation of the virus *in vivo*, in normal immunocompetent animals. It turns out that in all the species where they have been found, syncytins have conserved this feature from their ancestral infectious progenitor, at least one of them carrying a functional ISD in the case of primates and muroids where syncytins are present as pairs. This syncytin attribute is likely to be—or to have been—involved in maternal immune tolerance towards the semi-allogeneic feto–placental unit which expresses histo-incompatible paternal antigens. In fact, this feature could even constitute the primordial function of syncytins, prior to their fusogenic activity.

Indeed, the emergence of a primitive placenta in an oviparous ancestor may have been made possible thanks to the co-optation of a founding *env* gene that would have kept the immunosuppressive capacity of its ancient viral progenitor, allowing 'grafting' of a fetus exhibiting 'foreign' antigens from the father within the mother's body (figure 5). Along this line, it will be of interest to investigate the presence of *syncytin*-like genes in the epitheliochorial placenta (found in horse and pig), a tissue where cell–cell fusion does not occur. If the above hypothesis is correct, such genes are also expected there, which would have lost their fusogenic activity or their cognate receptor, and whose primary role would be immune protection of the fetus. Evidence for such a scenario could be provided by experiments—in progress—with genetically modified 'knock-in' mice in which the immunosuppressive function of the syncytins is specifically invalidated via introduction of appropriate point mutations that do not alter their fusogenic activity.

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