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Expression pattern of different gap junction connexins is related to embryo implantation

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ABSTRACT Successful implantation in mammals requires a close interaction between the embryo and the uterus. Direct cell-cell communication via gap junctions seems to play an important role in the preparation of the uterus for embryo implantation and in the regulation of trophoblast invasion. During preimplantation in the rat the gap junctional proteins connexin (cx) 26 and cx43 are suppressed. This loss of cell-cell communication seems to be important for transformation of the endometrium into the receptive phase. The suppressive effect is mediated by progesterone as demonstrated by the application of antigestagens. At implantation, however, a spatial and temporal pattern of connexin expression is induced in response to embryo recognition. cx26 is locally expressed in the uterine epithelium of the implantation chamber, cx43 in the surrounding decidua prior to invasion. With progressing invasion, the decidual cells surrounding the invading trophoblast in addition to cx43 reveal cx26. In this phase, the invasive partner, the blastocyst, is characterized by coexpression of cx43 and cx31. During trophoblast invasion however, cx31 becomes restricted to the cells of the invasive ectoplacental cone, cx43 to the embryo proper. It seems that compartmentalization of the trophoblast and the inner cell mass is established by two different connexins. During placental differentiation connexin expression switches from cx31 to cx26 and cx43, indicating the end of the invasive phase. The highly regulated pattern of connexin expression in the endometrium as well as in the trophoblast suggests a key role of this different intercellular pathways in regulating the invasion process of the trophoblast into its host tissue, the endometrium.

KEY WORDS: *gap junctions, connexins, trophoblast, endometrium, hormones*

Introduction

In mammals an intimate contact between embryo and mother is needed for successful implantation. Though the modus of implantation is highly divergent among species, all invasive types of implantation share the same cell biological process of trophoblast apposition, adhesion and its penetration into the endometrium (Schlafke and Enders, 1975). The cell biological analysis of this critical period requires the study of independent events in the embryo and the mother and also of interactions between both partners. The endometrial changes involved in the establishment of pregnancy including the pre- and periimplantation phase are governed by steroid hormones (Psychoyos, 1976, 1992). Ovarian steroids change the uterine epithelium from a nonadhesive state to the so-called "receptive phase" which allows the adhesion and invasion of the trophoblast (Schlafke and Enders, 1975; Schlafke *et al.*, 1985; Denker, 1990). In this initial phase the highly invasive trophoblast penetrates into the uterine stroma to arrode the maternal vessels and to establish a hemochorial placenta. This behavior resembles that of malignant tumor cells. However, in contrast to tumor cells trophoblast invasion is strictly controlled by the uterine environment indicating the close interaction of both partners. Local interactions between embryo and endometrium are already described (Dey and Johnson, 1986) and embryonic signals modulating the uterine environment seem to be necessary for initiation and regulation of implantation (for review see Kennedy, 1983).

Intercellular communication via gap junctions, which is involved in cell differentiation, seems to play an important role in preparing the uterus for embryo implantation as well as in the control of trophoblast invasion. Gap junctions are channels which connect the cytoplasms of neighbouring cells and allow ions and small molecules to pass from one cell to another, thereby coupling the cells both electrically and metabolically (reviews: Loewenstein, 1988; Bennett *et al.*, 1991). Each hemichannel is formed by six proteins (connexins) radially arranged around the pore (Fig. 1). About 15 different connexins, which all belong to a multigene family, have already been cloned (Beyer *et al.*, 1990; Willecke *et al.*, 1991; Haefliger *et al.*, 1992) and their sequences show very high identity between the species investigated. Although different unit conductances for gap junction channels comprised of different connexins have been recorded (rev: Kolb

Abbreviations used in this paper: dpc, days post coitum; cx, connexin.

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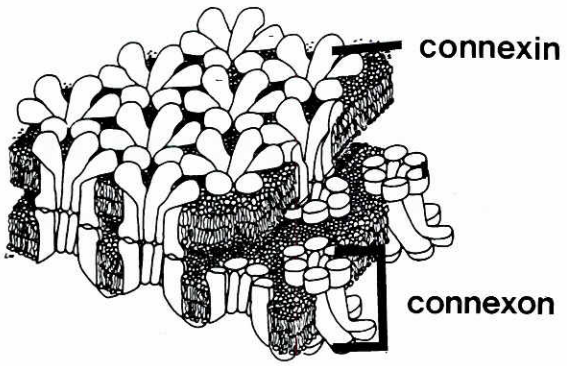


Fig. 1. Schematic drawing of gap junctional channels. *Each hemichannel (connexon) is composed of six protein subunits (connexins) (Makowski et al., 1977).*

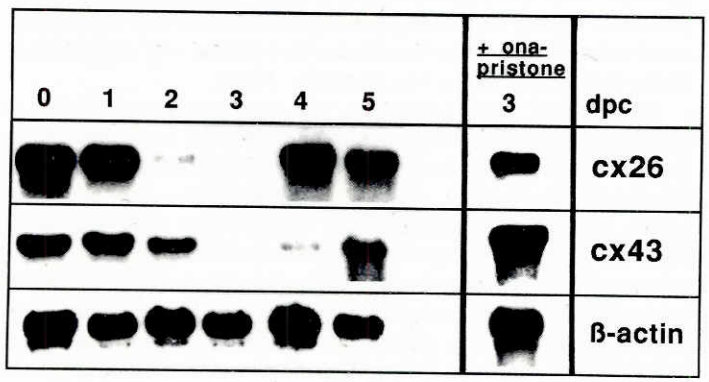
and Somogyi, 1991; Ramanan *et al.*, 1993), the physiological role of those channels still remains unknown. Thus the expression pattern of various connexin genes may be related to different stages of cellular differentiation and function.

In the recent years we have focused our interest on gap junction expression in both compartments, in the developing embryo as well as in the endometrium, during the pre- and periimplantation phase as a cell biological marker for their differentiation during trophoblast adhesion and invasion into the maternal tissue.

Connexin expression in the endometrium during pre- and periimplantation

Suppression of cell-cell communication during preimplantation

In the rat endometrium only two gap junctional connexins, cx26 and cx43, seem to play a role during the cyclic phases of nonpregnancy and during early pregnancy. In all cyclic phases of nonpregnancy cx26 as well as cx43 mRNA is evidenced in high amounts whereas the corresponding protein is rarely found (Winterhager *et al.*, 1993). During endometrial transformation to the "receptive phase" both connexin transcripts are completely suppressed in the endometrium of the preimplantation phase (Fig. 2a,b) (Grümmer *et al.*, 1994). This suppression of cx26 and cx43 and thereby the loss of direct cell-cell communication is under control of maternal progesterone. Applying the antiprogestin onapristone (ZK 98 299) on the first 2 or 3 days of pregnancy, a withdrawal of the connexin suppression can be observed (Fig. 2a), showing cx26 expression in the uterine epithelium (Fig. 2c) and a cx43 staining in the stromal cells in the preimplantation phase (Grümmer *et al.*, 1994). The influence of maternal steroid hormones on connexin expression in the endometrium could also be evidenced in ovariectomized rats treated with 17-ß-estradiol and/or progesterone. Untreated rats show cx43 mRNA, but no transcript for cx26. Estradiol drastically increases levels of both cx26- and cx43-mRNA whereas application of progesterone leads to a clear decrease of connexin transcripts. When estradiol and progesterone are administered simultaneously, the suppressive effect of progesterone predominates and no connexin expression can be found. Thus connexin expression in rat endometrium is regulated by maternal steroid hormones: Cx26 and cx43 are suppressed by progesterone and enhanced by estradiol. The suppressive effect of progesterone leads to a noncoupled endometrium during early pregnancy. Since nearly all tissues exhibit cell-cell communication, it is a phenomenon that the uterine epithelium is noncoupled during the preimplantation phase. This seems to be very important in regard to differentiation to a receptive epithelium as application of antiprogestins during this first days of pregnancy inhibit embryo implantation in rats and guinea pigs (Elger *et al.*, 1986; Roblero and Croxatto, 1991).



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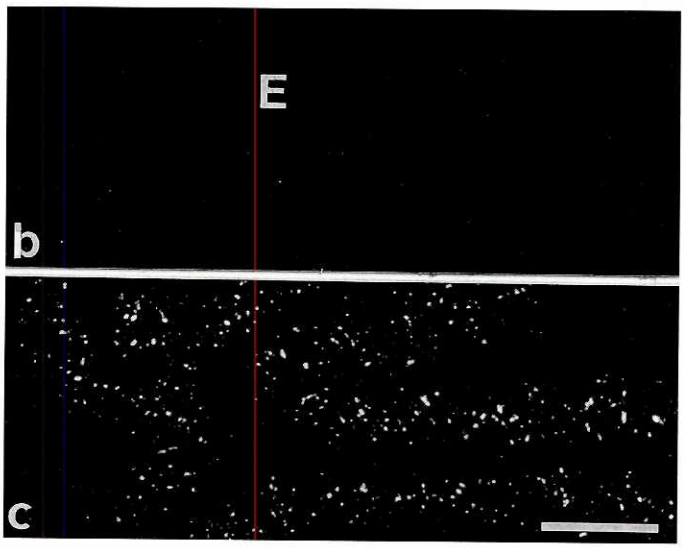

Fig. 2. Connexin expression in the rat endometrium during the periimplantation phase. (a) *Northern blot analysis of rat endometrial RNA from 0-5 dpc probed for cx26 and cx43 mRNA. In the preimplantation period (1-3 dpc) levels of mRNA for both connexins decline. At implantation (4 dpc) mRNA levels increase for both connexins. Onapristone treatment of pregnant rats on 0-2 dpc inhibited the decrease in connexin-mRNA levels observed on 3 dpc in untreated rats.* **(b,c)** *Immunohistochemical staining of cx26 in the rat endometrium on day 3 pc.* **(b)** *No staining of cx26 can be observed in the uterine epithelium (E).* **(c)** *Rats treated with onapristone on 0-2 dpc show a strong staining for cx26 in the luminal epithelial cells. Bars, 70 µm.*

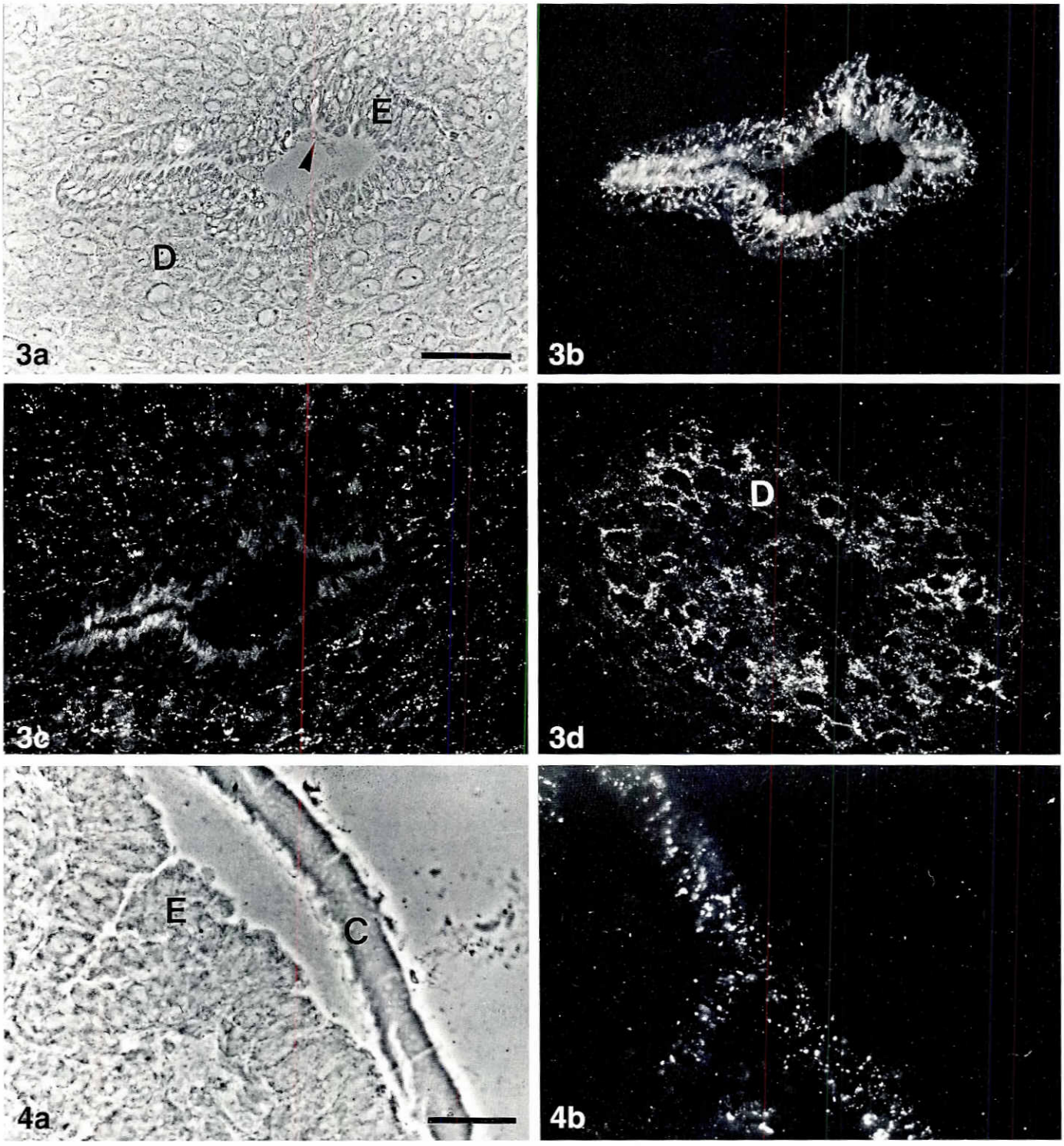

Fig. 3. Immunohistochemical staining of cx26 and cx43 in the rat implantation chamber. (a) *Phase contrast micrograph showing the trophoblast (arrowhead) surrounded by the uterine epithelium (E) on 5 dpc. The stroma has differentiated into decidual cells (D).* **(b,c)** *Same implantation chamber as shown in* **(a)**. *Cx26 is strongly expressed in the epithelium surrounding the blastocyst* **(b)**, *cx43 in the decidual cells* **(c)**. **(d)** *Immunohistochemical staining of cx26 in the implantation chamber on 7 dpc. The cx26-antigen is located in the decidual cell population (D) accompanying the invading trophoblast. Bars, 100 μm.*

Fig. 4. Immunohistochemical staining of cx32 in the rabbit implantation chamber on 6 dpc. (a) *Phase contrast micrograph shows that the blastocyst, still surrounded by the coverings (C), is attached to the uterine epithelium (E).* **(b)** *Cx32 is strongly expressed in the epithelial cells of the implantation chamber. Bars, 40 μm.*

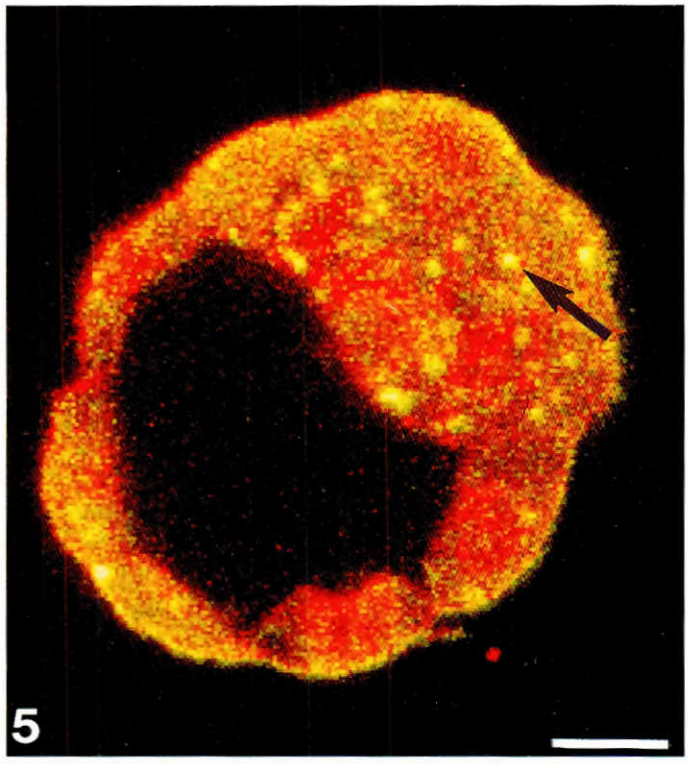

Fig. 5. Double immunofluorescence analysis of cx31 and cx43 in the 4.5 dpc blastocyst using confocal laser scan microscopy. *Green spots represent cx31, red spots cx43. Yellow spots indicate coexpression of both connexins in one gap junctional plaque (arrow). Cx31 and cx43 are evenly distributed throughout the trophoblast and the inner cell mass. Bars, 15 µm.*

A similar phenomenon could be evidenced for another species, the noncycling rabbit (Winterhager *et al.*, 1988). Here the endometrial epithelium stays noncoupled during nonpregnancy as well as during the preimplantation phase up to day 6 pc. In contrast to the rat, however, this suppression of cell-cell communication is not under control of ovarian steroid hormones and cannot be abolished by antiprogestins (Grümmer *et al.*, 1993). Thus suppression of direct cell-cell communication seems to represent an important precondition for preparing the endometrium for implantation though the mechanisms leading to this suppression seem to be different among species.

Induction of cell-cell communication in response to embryo recognition

As mentioned above suppression of intercellular communication is obviously necessary during the preimplantation period while the endometrium transforms to a receptive stage. This situation changes with the beginning of the implantation reaction: There is a clear induction of gap junction connexins in the endometrium in response to embryo recognition (Winterhager *et al.*, 1988, 1993). In rats situation changes on 5 dpc when implantation starts (Winterhager *et al.*, 1993). At this time cx26 is locally induced in the epithelium of the implantation chamber (Fig. 3a,b). Concomitantly decidualization of the stromal cells surrounding the implantation chamber starts as a reaction to the implanting blastocyst and is accompanied by the expression of cx43 (Fig. 3c). Thus prior to trophoblast invasion induction of cx26 is evidenced locally in the surrounding epithelium and cx43 in the developing decidua. With progressing invasion the epithelium degenerates by apoptosis (Welsh and Enders, 1991) and the trophoblast directly contacts the decidua. From this stage onwards in addition to cx43 immunostaining of cx26 is detected exclusively in the decidual cell population in the vicinity of the blastocyst (Fig. 3d). Thus at implantation a spatial and temporal pattern of connexin expression can be observed in response to embryo recognition in the rat.

Although the implantation modus between rat and rabbit is quite different (Schlafke and Enders 1975) a similar phenomenon can be seen in the rabbit. Here cell-cell communication via cx32 is locally induced in the uterine epithelium prior to invasion as a response to embryo recognition (Fig. 4 a,b) (Winterhager *et al.*, 1988; Grümmer *et al.*, 1993). This supports the idea that in a preconditioned uterus embryonic signals seem to trigger this differentiation step prior to trophoblast invasion (Winterhager *et al.*, 1988). Different hormones, second messengers, growth factors, mediators like prostaglandins, and even mechanical manipulations have been suggested to act as embryological signals (for review see DeFeo, 1967; Kennedy, 1983, 1985). Testing some of them in the rabbit uterus we could show that induction of cx32 transcripts in the uterine epithelium could be evoked by a mechanical stimulus and prostaglandins (Grümmer *et al.*, 1993). Similarly, in the rat a traumatic stimulus, given to a hormonally preconditioned uterus, induces decidualization and is accompanied by expression of cx43 and cx26 (Grümmer *et al.*, 1994). Interestingly, cx26 is restricted to those decidual cells around the traumatic stimulus leading to a similar distribution as observed during pregnancy.

Connexin expression in the preimplantation embryo and during trophoblast invasion

As differentiation of the embryo should be in relation to the differentiation of the endometrium, we investigated the spatial and temporal pattern of connexin expression in the rat trophoblast during the phases of early pregnancy.

It is well known that in the mouse preimplantation embryo cx43-mRNA expression starts from the 4-cell stage onwards leading to a well coupled 8-cell stage (Valdimarsson *et al.*, 1991; Ruangvoravat and Lo, 1992; De Sousa *et al.*, 1993). It was shown that the establishment of intercellular communication between the blastomeres is a precondition to maintain compaction (Lee *et al.*, 1987). We found that in addition to cx43 another connexin gene, cx31, is expressed in the preimplantation rat embryo in a similar spatial and temporal pattern as described for cx43 (Reuss *et al.*, 1996). Antigen of cx31 was detected in the 8-cell stage and a bright punctate staining for cx31 was found in the 4.5 dpc blastocyst. Double immunolabeling for cx43 and cx31 revealed that both connexins are mainly expressed in the same gap junctional plaque and are evenly distributed in the inner cell mass and the trophectoderm (Fig. 5). This situation changes immediately after implantation. We observed a compartmentalization of those two connexin transcripts. Cx31-mRNA and -protein now are expressed in the cells of the ectoplacental cone which invades into the maternal decidual tissue (Fig. 6a) where-

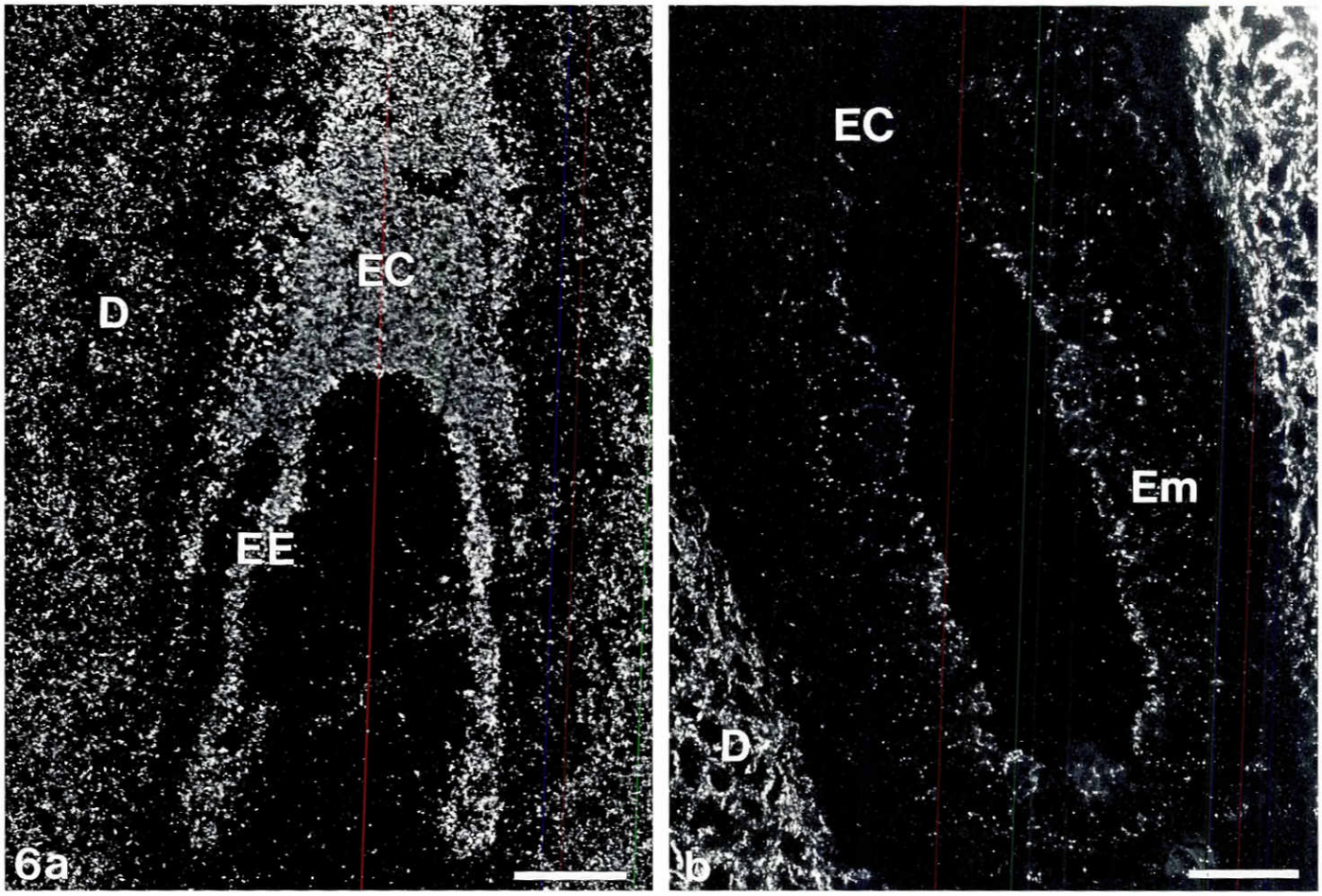

Fig. 6. Rat embryo on 9 dpc. (a) *In situ hybridization shows that cx31-mRNA is expressed in the ectoplacental cone (EC) and the extraembryonic ectoderm (EE).* **(b)** *Immunohistochemistry reveals a staining of cx43 in the embryo proper (Em), whereas the ectoplacental cone lacks cx43. (D, decidua). Bars: a, 150 µm; b, 100 µm.*

as the embryo proper is characterized by cx43 (Fig. 6b). This expression pattern correlates with the communication compartments between extraembryonic tissue in the mouse embryo described by Kalimi and Lo (1989). The establishment of such communication restriction borders could be due to the incompatibility of the cx31 and cx43 hemichannels (Elfgang *et al.*, pers. communication). As cx31 is the only connexin of the ectoplacental cone up to day 10 of pregnancy it seems to be the characteristic gap junctional protein during the invasive phase of the trophoblast. In this phase the highly differentiated decidua regulates the invasion of these cells (see above).

Placental differentiation should be shortly mentioned as it is involved in the termination of trophoblast invasion into the maternal tissue. The switch from the invasive phase to the differentiation phase is indicated by the induction of cx26 and cx43. Cx26 protein is expressed from day 12 pc onwards in amazing amounts in the labyrinthine trophoblast which is responsible for the feto-maternal exchange. Cx31 remains in the still proliferative spongiotrophoblast and is coexpressed with increasing amounts of Cx43 on 14 dpc. Starting with the induction of cx43, cx31 decreases with increasing trophoblast differentiation and is not longer detectable in the mature placenta (Reuss *et al.*, 1996).

Conclusions

The physiological role of gap junctions on the maternal side for receptivity and regulation of invasion as well as on the embryonal side for invasion and its termination is poorly understood. The highly regulated temporal and spatial pattern of connexin expression in the endometrium as well as in the trophoblast suggest a key role of this different intercellular pathways in regulating the invasion process.

In contrast to a steady-state expression in other adult tissues gap junction expression in the uterus demonstrates a high plasticity due to various functions of this tissue during the short period of pregnancy. This expression of different connexins is regulated by steroid hormones, and the suppression of connexin expression during the pre-implantation phase seems to be important for the establishment of the "receptive phase" in the species investigated. We found that the hormonal regulated connexin suppression and the blastocyst triggered connexin induction in the endometrium seems to be involved in regulating the implantation process during early pregnancy.

The induction of connexins in the endometrium is correlated to trophoblast invasion into the maternal endometrium. One

reaction of the endometrium to trophoblast invasion is the process of decidualization, where the stromal cell program changes (DeFeo, 1967; Glasser and Clark, 1975). This hints to a potential role of the decidual cells in actively regulating and directing trophoblast invasion. Without this regulation by the endometrium, e. g. when the trophoblast is transplanted to ectopic sites, it seems to be even more invasive than tumor cells (Solomon, 1966; Kirby, 1971; Sherman and Wudl, 1976). One step in this differentiation process of the decidua is the establishment of extensive intercellular communication and a local specialization of those cells surrounding the blastocyst which express cx26. We suppose that this connexin expression could function to establish or maintain coordinated differentiation of decidual cells, especially when those are penetrated by highly invasive trophoblast cells.

A precondition for trophoblast invasion and inner cell mass differentiation is the compartmentalization of these tissues. Compartmentalization into the embryo proper and the extraembryonic tissues starts after implantation and is mediated by two different connexins which are not able to establish a functional channel with one another. It remains to be clarified if this separation into two communication compartments is induced by the maternal environment. A special connexin, cx31, characterizes the proliferative and invasive phase of the trophoblastic cells which ends up by trophoblast differentiation indicated by increasing amounts of cx26 and cx43. Our studies indicate that the different connexin genes seem to be an excellent marker to indicate endometrial receptivity, blastocyst-maternal interactions and the state of trophoblast differentiation. The physiological role of those different channels, however, remains to be elucidated.

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