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Uterine morphology during diapause and early pregnancy in the tammar wallaby (Macropus eugenii).

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Running page heading: Uterine changes during diapause in Macropus eugenii

Abstract

In mammals, embryonic diapause, or suspension of embryonic development occurs when embryos at the blastocyst stage are arrested in growth and metabolism. In the tammar wallaby (Macropus eugenii), there are two separate uteri, only one of which becomes gravid with the single conceptus at a post-partum oestrus, so changes during pregnancy can be compared between the gravid and non-gravid uterus within the same individual. Maintenance of the viable blastocyst and inhibition of further conceptus growth during diapause in the tammar is completely dependent on the uterine environment. Although the specific endocrine and seasonal signals are well established, much less is known about the cellular changes required to create this environment. Here we present the first detailed study of uterine morphology during diapause and early pregnancy of the tammar wallaby. We combined transmission electron microscopy and light microscopy to describe the histological and ultrastructural changes to luminal and glandular epithelial cells. At entry into diapause after the post-partum oestrus and formation of the new conceptus, there was an increase in abundance of

organelles associated with respiration in the endometrial cells of the newly gravid uterus, particularly in the endoplasmic reticulum and mitochondria, as well as an increase in secretory activity. Organelle changes and active secretion then ceased in these cells as they became quiescent and remained so for the duration of diapause. In contrast, cells of the non-gravid, post-partum, contralateral uterus underwent sloughing and remodeling during this time and some organelle changes in glandular epithelial cells continued throughout diapause, suggesting these cells are not completely quiescent during diapause, although no active secretion occurred. These findings demonstrate that diapause, like pregnancy, is under unilateral endocrine control in the tammar, and that preparation for and maintenance of diapause requires substantial changes to uterine endometrial cell ultrastructure and activity.

Keywords: diapause, tammar, transmission electron microscopy, uterus, endometrium

Introduction

Embryonic diapause is a remarkable reproductive strategy in which normal pregnancy is interrupted by a period of suspended embryonic development (Renfree & Shaw, 2000). Extension of the gestation period has important life history consequences for diapausing species because successful pregnancy is ensured by allowing young to be born at optimal times of the year (Mead, 1993; Renfree & Shaw, 2000; Lopes et al. 2004). The selective advantage conferred by embryonic diapause is reflected by its widespread occurrence in mammals (Renfree & Shaw, 2000). Seven mammalian orders have diapause (Renfree & Calaby, 1981; Mead, 1993). The widespread distribution of diapause in mammals suggests it has evolved repeatedly (Mead, 1993; Renfree & Shaw, 2000; Fenelon et al., 2014; but see also Ptak et al. 2012).

Diapause traits also vary widely between mammalian groups, and even between closely related species (Mead, 1993; Renfree & Shaw, 2000; Fenelon et al., 2014). For example, the embryos of many species, including rodents, shed the zona pellucida and contact maternal cells before diapause, while those of marsupials and carnivores retain their acellular embryonic coats (Lopes et al. 2004). Embryonic development completely ceases during diapause in most species, yet some still exhibit slow cell growth throughout diapause (Renfree & Shaw, 2000; Lopes et al. 2004). The controls of diapause are diverse and diapause can either be facultative (i.e. controlled by

environmental factors or induced experimentally) as occurs in rodents and marsupials, or obligate (present in every gestation), as occurs in mustelid carnivores, the roe deer and in marsupials (Lopes et al. 2004). There is also great variation in duration of developmental arrest: from 4-10 days in rats and mice to 11 months in the tammar wallaby Macropus eugenii (reviewed in Renfree & Shaw, 2000).

Despite wide variation in diapause characteristics, embryonic development is arrested at the unilaminar blastocyst stage in all mammalian species that have diapause (Renfree & Calaby, 1981; Mead, 1993; Lopes et al. 2004). Recent evidence suggests that the molecular patterns involved in diapause, including the uterine molecular mediators Msx1 and Msx2, may also be highly conserved across unrelated groups of diapausing mammals (Cha et al., 2013). The uterus induces arrest of embryonic development and maintains viability of the embryo during quiescence (Lopes et al. 2004; Renfree & Shaw, 2014). Secretory activity of the uterus is typically tightly linked to activity of the corpus luteum and its secretion of progesterone, and the uterine environment is created by secretions from glandular and luminal epithelial cells during early pregnancy (Renfree & Shaw, 2000). Hence, pre-diapause uterine changes are the key to understanding how the uterus prepares for and maintains diapause. Uterine changes after reactivation of the embryo are tightly correlated with the ultrastructure of uterine cells and activity of both the uterus and the corpus luteum (Enders, 1967; Mead, 1993; Renfree & Shaw, 2000). However, the period leading up to diapause is poorly studied, and little is known of the ultrastructural changes associated with initiation of diapause (Renfree & Shaw, 2000; Lopes et al. 2004).

Diapause studies have typically used the mouse as a model in which diapause is induced experimentally (O'Neill & Quinn, 1983; Renfree & Shaw, 2000). However, the mouse embryo hatches from the zona pellucida before diapause, so identifying the role of the uterine environment in the initiation of arrest is confounded by the direct cellular contact with the maternal cells (Weitlauf, 1994; Renfree & Shaw, 2000). Marsupials are ideal models for such studies because they have a keratinous shell coat and thick mucin layer that prevents direct contact between embryonic and maternal cells. Thus from the time the embryo enters the uterus, through early pregnancy and diapause, and until placental attachment occurs only in the final third of pregnancy, the embryo is dependent on secretions from the uterine glands for nourishment (Renfree & Shaw, 2000).

The reproductive features of the tammar make this species ideal for studies of the initiation of diapause. Like all marsupials, tammar wallabies possess two completely separate uteri, each with an associated ovary. The tammar is monovular, and since ovulation alternates between the two ovaries, only one uterus becomes gravid and carries an embryo (Renfree, 2000). Reproduction in the tammar is under both lactational and seasonal control (Tyndale-Biscoe & Renfree, 1987;

Renfree & Shaw, 2000). The annual breeding cycle of the tammar is highly predictable and is triggered by the summer solstice, December 22nd in the southern hemisphere. Births occur at the end of January and are followed by a post-partum oestrus (Tyndale-Biscoe & Renfree, 1987; Renfree, 1993) and mating normally occurs within an hour of birth (Rudd, 1994). Ovulation occurs from the ovary contralateral to the post-partum uterus the day after birth (Figure 1), and the embryo enters the uterus ipsilateral to this ovary (the newly gravid uterus) 1-2 days later. The embryo develops to the unilaminar blastocyst stage and diapause is initiated by the suckling stimulus of the pouch young. Both the corpus luteum and the embryo are held in arrest by the sucking stimulus until after the shortest day. Seasonal diapause intervenes after the winter solstice and the blastocyst and corpus luteum remain quiescent until the longest day when reactivation of the embryo and the corpus luteum occur (Tyndale-Biscoe & Renfree, 1987; Renfree, 1993; Renfree & Shaw, 2014).

Importantly, the anatomical arrangement of uteri and ovaries in the tammar means that each individual has an in-built 'control' uterus (Figure 1; Renfree, 2000). Both uteri (gravid and non-gravid) are under the same peripheral (systemic) endocrine conditions, but experience different local, unilateral, effects due to proximity to the developing follicle on one side or the corpus luteum on the other (Towers et al. 1986; Renfree & Blanden, 2000; Shaw & Renfree, 2006) or due to the presence of a developing embryo in pregnancy (Renfree, 1972). Thus comparison of the two uteri enables identification of the uterine changes that occur leading up to, and during, diapause.

Here we identify the changes in uterine endometrial cell morphology in both the newly gravid and newly non-gravid uterus (post-partum uterus; Figure 1) required for entry into embryonic diapause in the tammar wallaby. We combine transmission electron microscopy and light microscopy to identify histological and ultrastructural changes in both glandular and luminal epithelial cells.

Materials and Methods

Animals

Tammar wallabies of Kangaroo Island, South Australia origin were held in our marsupial colony of The University of Melbourne, Victoria, Australia or collected from Kangaroo Island, South Australia. Their diet in captivity consisted of pasture supplemented with lucerne cubes and water ad libitum. All animal handling and experimentation were approved by the University of Melbourne Animal Experimentation and Ethics Committees.

Collection of Uterine Tissues

Tissue from both gravid and non-gravid uteri was collected from wild pregnant tammar wallabies shot on Kangaroo Island, South Australia (n=25) or from pregnant females held at the breeding colony, University of Melbourne, Victoria (n=15) as previously described (Renfree & Tyndale-Biscoe 1978). The stage of development of the tissue was determined based on the developmental stage of the embryo and the age of pouch young using published growth curves (Poole et al. 1991) or from a known time after a detected birth or mating. The day of birth was designated as day 0 post-partum. Uterine tissue was collected from culled animals immediately after parturition (day 0) until day 8 post-partum, and from stages during early (20-50 days post-partum), mid (60-100 days post-partum) and late embryonic diapause (>150 days post-partum). The reproductive status of the uterus in each female was confirmed after careful examination of the ipsilateral ovary for the presence of a mature follicle, ruptured follicle or new corpus luteum and upon recovery of an embryo from either the oviduct or uterus, depending on the stage of development.

Full thickness strips of uterine tissue, endometrium and myometrium, of a longitudinal section of the same width were collected from both the newly gravid uterus and the post-partum uterus in all animals to eliminate regional variation. The endometrial layer consists of the luminal epithelium (a single layer of epithelial cells which make up the inner lining of the uterus and comes into direct contact with the embryo), the uterine or endometrial glands (tubules lined by secretory epithelial cells), and the connective tissue stroma. Each stage of development was represented by a minimum of three samples, and at least four longitudinal strips were collected from both uteri per animal for histological and ultrastructural analysis.

Light Microscopy of Uterine Tissues

Strips of uterine tissue were fixed fresh in 4% paraformaldehyde (PFA) overnight and then washed and stored in 70% ethanol. Samples were embedded in paraffin for light microscopy. Paraffin blocks were serially sectioned at 8 μm on a microtome and mounted onto gelatin-coated glass slides. Sections were cleared with histolene (Pathtech Diagnostics, Box Hill, Victoria), rehydrated through a series of ethanol concentrations and stained with Harris's haematoxylin and Putt's eosin. Sections were examined and photographed on a Leitz compound light microscope. At least four full thickness strips of uterine tissue from different blocks were examined from each gravid and non-gravid uterus per animal. The thickness of the luminal epithelium and endometrial stroma was measured following the approach of Cruz and Selwood (1997): thickness was measured along a transect perpendicular to the parallel proximal and distal surfaces of the luminal epithelium and endometrial stroma respectively. The abundance of uterine glands in the endometrium was estimated by counting the number of cross-sections of glands ("whole gland profiles") within three

0.5 mm squares for each animal. Whilst oedema of the stroma could affect the number of observed cross sections, it does not occur to any great extent until around day 15 of pregnancy (Renfree and Tyndale-Biscoe, 1973) and these samples were all taken before day 8.

Electron Microscopy of Uterine Tissues

For transmission electron microscopy (TEM), full thickness pieces (1 mm^3) were fixed fresh in 5% glutaraldehyde-paraformaldehyde overnight at 4°C and then stored in 0.1 M sodium cacodylate buffer until processing. Tissues were post-fixed in 1% osmium tetroxide (OsO_4) in 0.2 M cacodylate buffer for 1-2 hours at room temperature, stained with uranyl acetate in maleate buffer, dehydrated slowly through a series of ethanol concentrations from 30% to 100%, and then fully dehydrated in 100% dry ethanol for 30 minutes. Samples were transferred to epoxy propane and then to a combination of epoxy propane with an increasing ratio of Epon Araldite resin. The epoxy propane was allowed to evaporate from the solution under a fume hood. Finally, tissues were embedded in pure fresh Epon Araldite resin and incubated at 60°C for up to 48 hours. The resin consisted of a mixture of 25 mL Procure 812, 15 mL Araldite 502, 55 mL dodecenyl Succinic Anhydride (DDSA) and 1.25 mL benzyldimethylamine catalyst (BDMA) (ProSciTech, QLD, Australia). At least four samples from the post-partum and contralateral uteri were embedded for each animal.

Semi-thin sections of uterine tissue were cut at 1 μm with a glass knife on a Reichert-Jung Ultracut E ultramicrotome and stained with 1% toluidine blue. Sections were examined under a light microscope to confirm the presence and correct orientation of the luminal epithelium and glandular endometrium in each block. The muscular myometrial layer was excluded from sectioning. Thin sections were then cut at 70 nm with a diamond knife on the ultramicrotome and mounted on copper grids. Sections on grids were post-stained with 3% uranyl acetate for 5 min and 0.6% Reynold's lead citrate for 10 min. Images from at least four separate areas on each micrograph were collected with a Phillips-CM 10 transmission electron microscope.

Statistical analysis

All measurements are expressed as the mean ± standard error (s.e.m) at each stage of development. Differences between post-partum and contralateral uteri at the same stage were analysed using Student's t-tests and differences between stages were determined using analysis of variance (ANOVA). Significance was determined where $P < 0.05$. Analyses were conducted using SYSTAT 9.01 software (Richmond, CA, USA).

Results

Summary of major reproductive events

To assist in understanding the complex timing of events in each uterus preceding diapause in the tammar, a summary of both uterine and embryonic changes is presented as a timeline (Figure 2).

Mating occurs approximately 1 h after birth (on day 0; Rudd, 1994; Renfree & Shaw, 2014) which results in formation of a Graafian follicle in the contralateral ovary. Ovulation occurs 40 h after birth after a surge of oestradiol, and a single-celled embryo enters the uterus on day 2 post-mating and begins cleavage (reviewed in Renfree, 1994; Renfree & Shaw, 2014). By day 8, the sucking stimulus of the pouch young induces quiescence of the corpus luteum, and the embryo, by now a unilaminar blastocyst, enters into diapause (Renfree & Shaw, 2014). The major embryonic events are aligned with key changes in the uterus and corpus luteum in Figure 2. The uterine cellular changes involved in preparation for diapause in the tammar are described in the following paragraphs.

Histology of the newly gravid and post-partum uteri

Day of parturition (day 0)

Abundance of gland profiles was low in the stroma of the newly gravid uterus on the day of birth (Figure 3a), while gland profiles were significantly more abundant and tightly packed in the post-partum uterus (Figure 3f; 4a, $P < 0.01$). The luminal epithelium of the newly gravid uterus was significantly thicker than that of the post-partum uterus (Figure 3f; 4b, $P < 0.01$), while the endometrial stromal thickness was approximately 1.5 mm thick for both uteri (Figure 4c).

Day of ovulation (days 1-2)

A significant increase in gland profile abundance occurred between day 0 and day 1 in the newly gravid uterus (Figure 3b; 4a, $P < 0.01$) while a significant decrease in abundance occurred during this period occurs in the post-partum uterus (Figure 3g; 4a, $P < 0.01$). Luminal thickness increased significantly in both uteri between days 0 and 1, and was significantly thicker in the newly gravid uterus than the post-partum uterus (Figure 4b, $P < 0.01$).

Stromal thickness decreased significantly during this period in the post-partum uterus (Figure 4c, $P < 0.01$). After day 1, stromal thickness remained relatively constant in both uteri leading up to diapause, yet was significantly thicker in the newly gravid uterus than the post-partum uterus (Figure 4c, $P < 0.05$).

Early cleavage (days 3-5)

Gland profile abundance in both the newly gravid (Figure 3c) and post-partum uteri (Figure 3h peaked on day 5, and was significantly greater in the newly gravid uterus (90.3 ± 1.4 per 0.5 mm^2) than the post-partum uterus (68.9 ± 0.9 per 0.5 mm^2; Figure 4a).

The luminal thickness of the post-partum uterus was significantly thinner than that of the newly gravid uterus throughout early pregnancy and diapause (Figure 4b, $P < 0.05$) and remained relatively constant.

Cleavage and unilaminar blastocyst (days 6-8)

A rapid decrease in gland profile abundance in the newly gravid uterus occurred between days 6 and 8 in both the newly gravid (Figure 3d) and post-partum uterus (Figure 3i; 4a). By day 8, the abundance of gland profiles for both uteri had reduced to 40 - 45 per 0.5 mm^2 (Figure 4a).

Diapause

No changes in luminal cell histology occurred during diapause in either uterus. Gland profile abundance during diapause in both the newly gravid and post-partum uteri was similar to that of day 1 post-partum (Figure 3e; 3j; 4a), and significantly lower gland profile abundance occurred in the post-partum uterus during diapause than on day 0 (Figure 4a, $P < 0.01$). Both luminal (Figure 4b) and stromal thickness (Figure 4c) remained constant throughout diapause.

Ultrastructure of glandular epithelial cells of the newly gravid uterus

Day of parturition (day 0)

The cytoplasm of glandular epithelial cells contained a large well-developed Golgi body, lipid droplets (Figure 5a), and undilated strands of smooth endoplasmic reticulum (SER). The glandular lumen (GL) contained secreted material (Figure 5a).

Day of ovulation (days 1-2)

A marked change in gland cell ultrastructure occurred between days 0 and 1. Cells on day 1 contained large basal clusters of lysosomes and elongated mitochondria (Figure 5b). The GL was still filled with secretions at this stage, but secretory vesicles were fewer than previously, and even fewer were present by day 2 post-partum. Apical and central clusters of lipid droplets and dark granules appeared by this stage, and strands of rough endoplasmic reticulum (RER) were markedly dilated.

Early cleavage (days 3-5)

Gland cells possessed large secretory vesicles by day 3 (Figure 5c), which became more clustered by day 4 (not shown). Elongated mitochondria and dilated RER were prominent and lysosomes were particularly common in cells that were shedding into the lumen. The increase in gland profile abundance on day 5 was accompanied by an increase in gland cell organelles. Golgi bodies were extensive, and strands of RER and mitochondria were abundant in the cytoplasm (Figure 5c). The glandular lumen was completely filled with secreted material.

Cleavage and unilaminar blastocyst (days 6-8)

Glandular cells contained massively dilated channels of RER, and the glandular lumen was still filled with secretions and vesicles by this stage. The decrease in gland abundance by day 8 was followed by the apical accumulation of very large secretory vesicles. The gland cell cytoplasm was devoid of many organelles by this stage, although pale secretory granules and lipid droplets occurred occasionally (Figure 5d). The glandular lumen was filled with dense flocculent material.

Diapause

Gland cell ultrastructure was relatively consistent throughout diapause. Secretory vesicles were common, but there was no evidence of active secretion (Figure 5e). The cytoplasm contained an abundance of organelles, particularly elongated mitochondria and lysosomes. In contrast to previous stages, Golgi bodies were poorly developed and SER strands were undilated (Figure 5f).

Ultrastructure of glandular epithelial cells of the post-partum uterus

Day of parturition (day 0)

Gland profiles were abundant in the stroma on the day of birth and gland cells were rich in organelles, with very well-developed Golgi bodies, strands of SER and RER, and abundant small mitochondria (Figure 6a). Few secretory vesicles occurred, but the glandular lumen contained secreted material.

Day of ovulation (days 1-2)

Gland cells contained fewer organelles following the decrease in abundance of gland profiles between days 0 and 1 (Figure 6b). Golgi bodies were less well-developed and only small amounts of SER and scattered mitochondria are present. Lysosomes also increased in abundance. Little change in glandular cytology occurred between day 1 and day 2, but ribosomes became dispersed in the cytoplasm. The glandular lumen was mostly empty by this stage, and microvilli were sparse on the apical surface.

Early cleavage (days 3-5)

Degenerative cells with abundant lysosomes occurred in the glands of the post-partum uterus (Figure 6c). Lysosomes were also common in normal cells, and reached peak abundance by day 5 (Figure 6d). Golgi bodies were well developed and channels of RER were dilated. Secretory vesicles became less common by this stage.

Cleavage and unilaminar blastocyst (days 6-8)

Secretory vesicles began to increase by day 6, as gland profile abundance decreased, and pale vesicles were common by day 7 (Figure 6e). Gland cells on day 6 contained granular material, small dark granules, as well as well-developed Golgi bodies and dilated RER (not shown). Microvilli were sparse. Golgi bodies were less well-developed by day 7, and clusters of lysosomes were the most prominent cell features. Glycogen deposits were occasionally seen by day 8, and the GL appeared to be filled by material extruded by cells.

Diapause

In contrast to cells of the newly gravid uterus, gland cells of the post-partum uterus underwent some cellular change throughout diapause. Secretory vesicles were common in the cytoplasm by mid diapause (Figure 6f). Cell cytology was very similar between early and mid diapause, but electron-dense vesicles and SER became much more common by late diapause (Figure 6g-h). Few dark granules or glycogen deposits were present, and cells lacked microvilli. As for the newly gravid uterus, there was no evidence of cellular secretion into the GL.

Ultrastructure of luminal epithelial cells of the newly gravid uterus

Day of parturition (day 0)

Cuboidal epithelial cells possessed very large nuclei that fill most of the cytoplasm (Figure 7a). Other organelles were few, aside from occasional dark granules and lysosomes, suggesting the cells are relative inactive. Both microvillous and ciliated cells lined the uterus, and microvilli were slender.

Day of ovulation (days 1-2)

Luminal cells showed evidence of active exocytosis by day 1 (Figure 7b). The supranuclear region contained abundant mitochondria, lysosomes, and well-developed Golgi bodies. Microvilli were slender. Some degenerative cells occurred in the uterus by day 2.

Early cleavage (days 3-5)

Luminal cell cytology was similar between days 4-6. Many organelles occurred apically, including well-developed Golgi bodies, ovoid mitochondria (Figure 7c), and strands of both rough

and smooth endoplasmic reticulum. Microvilli were long and slender. Large secretory vesicles occurred in some cells.

Cleavage and unilaminar blastocyst (days 6-8)

The cell cytoplasm contained a fine scattering of dark glycogen granules (Figure 7d). Lipid droplets occurred throughout the cytoplasm, but secretory vesicles were rare. On day 8, apical microvilli were plump, and cells contained large basal mitochondria, well-developed Golgi, short strands of RER and spherical mitochondria near the apical surface. No secretory vesicles were present, but dark secretory granules occurred in the uterine lumen.

Diapause

As for gland cells, luminal cell morphology remained similar throughout early, mid and late diapause. Some ciliated cells occurred, and microvilli were sparse. The cytoplasm contained a moderately dense scattering of glycogen droplets, giving the cytoplasm a mottled appearance (Figure 7e). Large mitochondria and SER were common during diapause.

Ultrastructure of luminal epithelial cells of the post-partum uterus

Day of parturition (day 0)

Secretory vesicles, lysosomes and mitochondria were prominent in the cytoplasm of luminal cells (Figure 8a). Cell apices were covered with short, compact microvilli.

Day of ovulation (days 1-2)

Degenerative cells appeared in the post-partum uterus by day 1. Ciliated cells were more abundant than microvillous cells by this stage (Figure 8b). Cells contain fewer organelles than previously, but mitochondria were prominent near the basal and lateral membranes. Small glycogen deposits were present in microvillous cells, and ciliated cells possessed large ovoid nuclei with prominent nucleoli.

Early cleavage (days 3-5)

In contrast to luminal cells of the newly gravid uterus, secretory granules were prominent in the cytoplasm, and few organelles occurred apically, except for mitochondria (Figure 8c). By day 4, microvillous cells were more common than ciliated cells, but mitochondria were sparse on the

apical surface (Figure 8c). Few organelles occurred basally, but mitochondria were common in the supranuclear region and at the cell borders.

Cleavage and unilaminar blastocyst (days 6-8)

Luminal cell morphology was similar between days 5-6, and clusters of secretory vesicles appeared by day 7 (Figure 8d). Microvillous cells were more common than ciliated cells, but ciliated cells were more common on day 7 than in the newly gravid uterus. Ciliated cells contained abundant lysosomes and mitochondria, with a prominent supranuclear Golgi body. Microvilli were sparse and the basal cytoplasm of these cells contained dark granules, lipid droplets, and mitochondria (Figure 8d).

Diapause

Cells of the post-partum uterus were markedly different from those of the newly gravid uterus. Microvilli were more numerous, and lysosomes were more abundant. Mitochondria were smaller, and cells lack glycogen deposits. Cells had prominent well-developed Golgi complexes (Figure 8e). As found in the newly gravid uterus, there was no evidence of active secretion.

Discussion

Preparation for diapause in the tammar wallaby involved significant changes in cell ultrastructure and activity. Both luminal and glandular epithelial cells of the newly gravid uterus underwent changes in organelle abundance and distribution in early pregnancy, which were accompanied by an increase in secretory activity. Re-organisation also occurred in cells of the post-partum uterus, although cells were markedly different from those of the newly gravid uterus. This difference was most pronounced during diapause as cells of the newly gravid uterus were completely quiescent, while some organelle changes continued in the post-partum uterus.

Morphology of the gravid tammar endometrium changed dramatically within a day of birth (between day 0 and day 1 post-partum) and around the time of the post-partum oestrogen pulse. Gland profile density and gland cell organelle abundance increased during this period, and previously inactive luminal cells underwent organelle changes and began active exocytosis. Luminal cell morphology on this day was similar to that during oestrus of the brushtail possum (Trichosurus vulpecula; Shorey & Hughes, 1973) and of women (Ludwig & Metzer, 1976). Dramatic remodeling of luminal cell morphology also occurs in a wide range of eutherian mammal (reviewed in Murphy, 2004; 2010) and marsupial species (Laird et al., 2014; 2015; Dudley et al.,

2015) in preparation for implantation of the embryo. The ultrastructural changes in the tammar suggest an increase in secretion and packaging processes in the cytoplasm, and thus an increase in cell activity, occurs during this period, most likely in response to oestradiol released from the developing follicle (Renfree and Shaw, 2000).

Cellular activity in the newly gravid uterus increased as pregnancy progressed. Luminal epithelial cells possessed well-developed Golgi complexes, secretory vesicles and large mitochondria by day 3 post-partum, following entry of the embryo in the uterus on day 2. Notably, a significant increase in gland profile density occurred on days 5 and 6 post-partum, followed by massive dilation of both rough and smooth endoplasmic reticulum and an increase in abundance of mitochondria. These organelle changes indicate an increased rate of cell respiration and protein synthesis. In addition, secretory vesicles reach peak abundance by days 5 and 6 post-partum as the sucking of the pouch young begins to induce the onset of quiescence of the corpus luteum (Tyndale-Biscoe, 1986).

Importantly, uterine gland cell activity decreased just before the onset of diapause. Both organelle abundance and secretory activity of gland cells decreased by day 8 post-partum, suggesting that preparation for diapause has already begun. This result is consistent with a decrease in total protein concentration and volume of secretions in the tammar during this period (Renfree, 1972; Renfree, 1973), as well as with a significant reduction in plasma progesterone at the onset of embryonic diapause, resulting from the inhibitory effect of the suckling stimulus of the pouch young on the corpus luteum (Renfreee and Shaw, 2000). As diapause is the result of a uterine environment that is inappropriate to support continuous growth of the embryo, yet maintains its viability (Mead, 1993; Lopes et al. 2004), the decrease in activity during this period in the tammar may represent the shift from supporting embryonic growth to preparing for quiescence.

The tammar embryo is surrounded by the shell coat and a permeable acellular mucin layer and a zona pellucida which prevent direct contact between maternal and embryonic cells until at least 18 days after termination of diapause (Denker and Tyndale-Biscoe, 1986; Renfree, 2000; Renfree & Shaw, 2000). In contrast, in species like the mouse, the embryo hatches from the zona before diapause and so may be influenced by both the uterine environment and direct maternal contact (Weitlauf, 1994; Renfree & Shaw, 2000). Thus, in tammar wallabies, significant changes in uterine ultrastructure may be important in regulation of diapause, but changes in the surface luminal epithelium cannot directly affect the embryo through cell-cell contact (Renfree & Shaw, 2000). While the uterine changes involved in diapause initiation in mice and rats are largely unknown, uterine changes shared by both rodents and the tammar in early pregnancy are likely to be essential

for diapause. However, changes that are unique to the tammar wallaby are likely to also be related to the complete dependence of the tammar wallaby on uterine secretions during diapause.

Cells of the newly gravid and post-partum uteri were markedly different at all stages of early pregnancy and diapause. Early pregnancy in the post-partum uterus was characterized by cell degradation and remodeling. Both lysosomes and degenerative cells were more common in the post-partum uterus and a significant decrease in both gland profile abundance and of organelles in gland cells occurred within a day of birth, suggesting that the tammar uterus may rapidly revert to the receptive state. Cellular debris was sloughed into the glandular lumen as pregnancy progresses, filling the glandular lumen by day 8 post-partum. Removal of degenerative material from the uterus and remodeling post-partum occurs in many mammalian species, including the rat (Png & Murphy, 1997), and the marsupials Sminthopsis crassicaudata (Laird et al. 2014) and Didelphis virginiana (Padykula & Taylor, 1975), as the uterus prepares to receive a new conceptus.

Cellular differences between the two uteri were most pronounced during diapause as cells of the newly gravid uterus were completely quiescent while some changes in organelle abundance occurred during diapause in those of the post-partum uterus. Quiescence in the newly gravid uterus is expected as activity of the uterus is tightly linked to the quiescent corpus luteum (Tyndale-Biscoe & Renfree, 1987; Renfree & Shaw, 2014). No active secretion occurs in either uterus during diapause and large secretory droplets accumulate in quiescent gland cells, while glycogen deposits accumulate in quiescent luminal cells, indicating a reduction in cell activity (Enders, 1967). Active secretion typically ceases during mammalian diapause (Daniel, 1971; Surani, 1975), and many species also develop secretory vesicles in gland cells, including the roe deer and mink (Enders, 1967), which are released after reactivation of the blastocyst (Daniel, 1971; Surani, 1975). Reactivation in the tammar, as in many mammalian species, follows a significant increase in secretory activity corresponding to a spike in plasma progesterone (Freyer et al. 2002; Hinds & Tyndale-Biscoe, 2013). Hence the accumulation of secretory vesicles in the tammar is likely to be in preparation for embryonic development following reactivation.

The anatomical and functional separation of the newly gravid and post-partum uteri in the tammar allows for comparison of responses of both within an individual throughout pregnancy. Since both uteri are under the same peripheral endocrine conditions, a local effect, rather than a peripheral endocrine effect, must be responsible for the structural changes of the uterus in the tammar and for the marked cellular differences between both uteri throughout early pregnancy and diapause. Hence these uterine changes are under unilateral control. Unilateral differences also occur between the pregnant and non-pregnant endometrium of other marsupial species, including Antechinus stuartii (Cruz & Selwood, 1993), Sminthopsis macroura (Cruz & Selwood, 1997) and

T. vulpecula (Shorey & Hughes, 1973), despite similar profiles of ovarian hormones in both cycles (Tyndale-Biscoe & Renfree, 1987). In the tammar, preferential delivery of oestradiol from the Graafian follicle to the newly gravid uterus at the time of birth results in an increase in both progesterone and oestradiol receptors in the newly gravid uterus relative to the post-partum uterus (Renfree & Blanden, 2000), priming the uterus to the subsequent action of progesterone (Renfree & Blanden, 2000). A similar unilateral increase in receptors also occurs in the quokka (Setonix brachyurus; Owen et al. 1982) and the brushtail possum (T. vulpecula; Curlewis & Stone, 1986). Hence differences in hormone receptor concentrations between uteri result in different uterine responses to the same endocrine conditions (Tyndale-Biscoe & Renfree, 1987) and may explain the observed unilateral effects in the tammar.

The findings of this study demonstrate that major changes in uterine cell ultrastructure and activity are involved in preparation for diapause in the tammar. Important changes required for tammar diapause, including cessation of active secretion and reorganisation of uterine cells, are consistent with major patterns across diapausing mammals. Yet the specific ultrastructural changes involved in this process vary remarkably between groups of diapausing mammals (Renfree & Shaw, 2000), and even within a genus (Mead, 1993). For example, accumulation of glycogen in quiescent glandular cells in the tammar also occurs in the majority of diapausing mammals. However, the amount of glycogen stored can be enormous, as in several mustelid species, while only a very small amount accumulates in another mustelid, the mink (Enders, 1967). In addition, many species also exhibit unique ultrastructural changes, such as the formation of unusually large spherical mitochondria in the mink (Enders, 1967). Wide variation in uterine cytology suggests that there may be no common pattern of change among species (Enders, 1967), and supports the theory that diapause has evolved repeatedly in mammals (Mead, 1993; Renfree & Shaw, 2000; Ptak et al, 2012). Shared uterine changes, both morphological and molecular, are likely to be essential for initiation of diapause and may constrain the evolution of diapause within mammals (Freyer et al. 2002; Cha et al., 2013) but cellular differences suggest that there may be some flexibility in the specific changes required for preparation for diapause across diverse mammalian groups.

Acknowledgments

Author contributions

GS, MBR and CMH collected the tissue used in this study. CMH undertook all technical work including tissue preparation and microscopy, prepared figures and interpreted the results. GS assisted with the figures. MKL prepared the manuscript. MBR and GS advised with experimental design and all authors assisted in manuscript preparation.

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The authors have no conflicts of interest to declare.

Reference list

Cha J, Sun X, Bartos A, Fenelon J, Lefèvre P, Daikoku T et al. (2013) A new role for muscle segment homeobox genes in mammalian embryonic diapause. Open Biol 3, 130035.

Cruz YP, Selwood L (1993) Uterine histology of the dasyurid marsupial, Antechinus stuartii: relationship with differentiation of the embryo. J Reprod Fertil 99, 237-242.

Cruz YP, Selwood L (1997) Histological differences between gravid and non-gravid uteri in the dasyurid marsupial, Sminthopsis macroura. J Reprod Fertil 111, 319-325.

Curlewis JD, Stone GM (1986) Effects of oestradiol, the oestrous cycle and pregnancy on weight, metabolism and cytosol receptors in the uterus of the brush-tail possum (Trichosurus vulpecula). J Endocrinol 108, 201-210.

Daniel JC (1971) Growth of the preimplantation embryo of the northern fur seal and its correlation with changes in uterine protein. Dev Biol 26, 316-322.

Denker H-W, Tyndale-Biscoe, CH (1986) Embryo implantation and proteinase activites in a marsupial (Macropus eugenii). Histochemical patterns of proteinases in various gestational stages. Cell Tissue Res 246, 279-291.

Dudley JS, Murphy CR, Thompson MB, McAllan BM (2015) Desmoglein-2 during pregnancy and its role in the evolution of viviparity in a marsupial (Sminthopsis crassicaudata; Dasyuridae). J Morphol 276, 261-272.

Enders AC (1967) The uterus in delayed implantation. In: Cellular Biology of the Uterus, (ed RM Wynn), pp. 151-190. North-Holland Publishing Company, New York.

Fenelon JC, Banerjee A, Murphy BD (2014) Embryonic diapause: development on hold. Int J Dev Biol 58, 162-174.

Freyer C, Zeller U, Renfree MB (2002) Ultrastructure of the placenta of the tammar wallaby, Macropus eugenii: comparison with the grey short-tailed opossum, Monodelphis domestica. J Anat 201, 101-119.

Hinds LA, Tyndale-Biscoe CH (2013) Daily prolactin pulse inhibits the corpus luteum during lactational quiescence in the marsupial, Macropus eugenii. Reprod, Fertil Dev 25, 456-461.

Laird MK, Thompson MB, Murphy CR, McAllan BM (2014) Uterine epithelial cell changes during pregnancy in a marsupial (Sminthopsis crassicaudata; Dasyuridae). J Morphol 275: 1081-1092.

Laird MK, Turancova M, McAllan BM, Murphy CR, Thompson MB (2015) Unlocking amniote live birth: the 'other' mammalian model. J Proc Roy Soc NSW 148, 52-59.

Lopes FL, Desmarais JA, Murphy BD (2004) Embryonic diapause and its regulation. Reproduction 128, 657-668.

Ludwig H, Metzger H (1976) The Human Reproductive Tract. Springer-Verlag, Berlin.

Mead RA (1993) Embryonic diapause in vertebrates. J Exp Zool 266, 629-641.

Murphy CR (2004) Uterine receptivity and the plasma membrane transformation. Cell Res 14(4), 259-267.

Murphy CR (2010) Comparative placentation, epithelial plasticity and the 'efficient barrier hypothesis'. Herp Con Bio 5(2), 320-323.

O'Neill C, Quinn P (1983) Inhibitory influence of uterine secretions on mouse blastocysts decreases at the time of blastocyst activation. J Reprod Fertil 68, 269-274.

Owen FJ, Cake MH, Bradshaw SD (1982) Characterisation and properties of a progesterone receptor in the uterus of the quokka (Setonix brachyurus). J Endocrinol 93, 17-24.

Padykula HA, Taylor JM (1975) Cellular mechanisms involved in cyclic stromal renewal of the uterus. I. The opossum, Didelphis virginiana. Anat Rec 184, 5-26.

Png F, Murphy CR (1997) The plasma membrane transformation does not last: microvilli return to the apical plasma membrane of uterine epithelial cells after the period of uterine receptivity. Eur J Morphol 35(1), 19-24.

Poole WE, Simms NG, Wood JT, Lubulwa M (1991) Tables for age determination of the Kangaroo Island wallaby (tammar), Macropus eugenii, from body measurements. CSIRO Division of Wildlife and Ecology, Technical Memorandum No. 31.

Ptak GE, Tacconi E, Czernik M, Toschi P, Modlinski JA, Loi P (2012) Embryonic diapause is conserved across mammals. PLoS ONE 7(3), e33027.

Renfree MB (1972) Influence of the embryo on the marsupial uterus. Nature 240, 475-477.

Renfree MB (1973) Proteins in the uterine secretions of the marsupial Macropus eugenii. Dev Biol 32 41-49.

Renfree, M.B. & Tyndale-Biscoe, C.H. (1973) Intrauterine development after diapause in the marsupial, Macropus eugenii. Dev. Biol. 32: 28-40.

Renfree MB, Tyndale-Biscoe CH (1978) Manipulation of marsupial embryos and pouch young. In Methods in

mammalian reproduction (ed. JC Daniel Jr), pp. 307-331. Academic Press, New York.

Renfree, MB, Calaby, JH (1981) Background to delayed implantation and embryonic diapause. J. Reprod. Fert. Suppl. 29, 1-9.

Renfree MB (1993) Diapause, pregnancy and parturition in Australian marsupials. J Exp Zool 266, 450-462.

Renfree MB (1994) Endocrinology of pregnancy, parturition and lactation of marsupials. In: Marshall's Physiology of Reproduction (ed. GE Lamming), pp. 677-766. Chapman & Hall, London.

Renfree MB (2000) Maternal recognition of pregnancy in marsupials. Rev Reprod 5, 6-11.

Renfree MB, Blanden DR (2000) Progesterone and oestrogen receptors in the female genital tract throughout pregnancy in the tammar wallabies. J Reprod Fertil 119, 121-128.

Renfree MB, Lewis AM (1996) Cleavage in vivo and in vitro in the marsupial Macropus eugenii. Reprod Fertil Dev 8, 724-42.

Renfree MB, Shaw G (2000) Diapause. Annu Rev Physiol 62, 353-75.

Renfree MB, Shaw G (2014) Embryo-endometrial interactions during early development after embryonic diapause in the marsupial tammar wallaby. Int J Dev Biol 58, 175-181.

Renfree MB, Frankenberg SR, Freyer C (2011) Trophoblast, placenta and early embryo: How the marsupial develops. Reprod Fertil Dev 22 (Supp.), 15-15.

Rudd CD (1994) Sexual behaviour of male and female tammar wallabies (Macropus eugenii) at post partum oestrus. J Zool 232, 151-162.

Shaw G, Renfree MB (2006) Parturition and perfect prematurity: birth in marsupials. Aust J Zool 54,139-149.

Shorey CD, Hughes RL (1973) Cyclic changes in the uterine endometrium and peripheral plasma concentrations of progesterone in the marsupial Trichosurus vulpecula. Aust J Zool 21, 1-19.

Surani MA (1975) Hormonal regulation of proteins in the uterine secretion of ovariectomized rats and the implications for implantation and embryonic diapause. J Reprod Fertil 43, 411-417.

Towers PA, Shaw G, Renfree MB (1986) Urogenital vasculature and local steroid concentrations in the uterine branch of the ovarian vein of the female tammar wallaby (Macropus eugenii). J Reprod Fertil 78, 37-47.

Tyndale-Biscoe CH (1986) Embryonic diapause in a marsupial: role of the corpus luteum and pituitary in its control. Prog Clin Biol Res 205, 137-155.

Tyndale-Biscoe CH, Renfree MB (1987) Reproductive physiology of marsupials. Cambridge University Press, Cambridge.

Weitlauf HM (1994) Biology of Implantation. In: The Physiology of Reproduction, Vol I, (eds E Knobil and JD Neill), pp 391-440. Raven Press Ltd., New York.

Figure legends

Figure 1: Reproductive cycle of the tammar wallaby (Macropus eugenii). The uterus associated with the foregone pregnancy is termed the post-partum uterus. The contralateral uterus which receives the embryo, and is associated first with the Graafian follicle, then with the new corpus luteum (CL) following ovulation, is termed the newly gravid uterus.

Figure 2: Summary of the key uterine changes during pregnancy and diapause in the tammar wallaby (Macropus eugenii) associated with changes in the ovary and embryo; Corpus luteum (CL), Golgi complex (G), Graafian follicle (G follicle), granulosa cells (GC), glandular epithelium (GE); luteal cells (LC); luminal epithelium of uterus (LE); mitochondria (M); rough endoplasmic reticulum (RER); smooth endoplasmic reticulum (SER); sperm (sp); secretory vesicles (SVes).

[1]Rudd (1994); [2]Renfree & Lewis (1996), Tyndale-Biscoe (1986), Renfree (1993); [3]Renfree et al, 2011.

Figure 3: Histological sections of the newly gravid and post-partum uteri of the tammar wallaby. The uterus is lined by the luminal epithelium (le), with uterine glands (gl) embedded in the connective tissue stroma (st) which is underlaid by myometrium (m). Sections are stained with haemotoxylin and eosin. A-E: Newly gravid uterus. A) Day 0 post-partum; B) Day 1 post-partum; C) Day 5 post-partum; D) Day 8 post-partum; E) Diapause. F-J: Post-partum uterus. F) Day 0 post-partum; G) Day 1 post-partum; H) Day 5 post-partum; I) Day 8 post-partum; J) Diapause; Scale bar = 100 μm.

Figure 4: Measurements of uterine tissues in the tammar wallaby from days 0-8 post-partum and in diapause (E= early, M= middle, L= late). A) Abundance of endometrial gland profiles (cross-sections) per 0.5 mm^2; B) thickness of the luminal epithelium; C) thickness of the endometrial stroma. Measurements (means ± s.e.m) are from both post-partum (open symbols) and newly gravid uteri (solid symbols) from day 0 to day 8 post-partum, and during embryonic diapause. Asterisks denote values that are significantly different from the day of parturition (day 0 post-partum), where * $P < 0.05$; ** $P < 0.01$. A timeline shows the stage of embryonic development relative to the time of birth: Ovulation (Ov); Embryo enters the uterus (Ut); Cleavage stage of the embryo in the uterus (2, 8, 16, 32 or 64 cells); formation of the blastocyst in the days after birth.

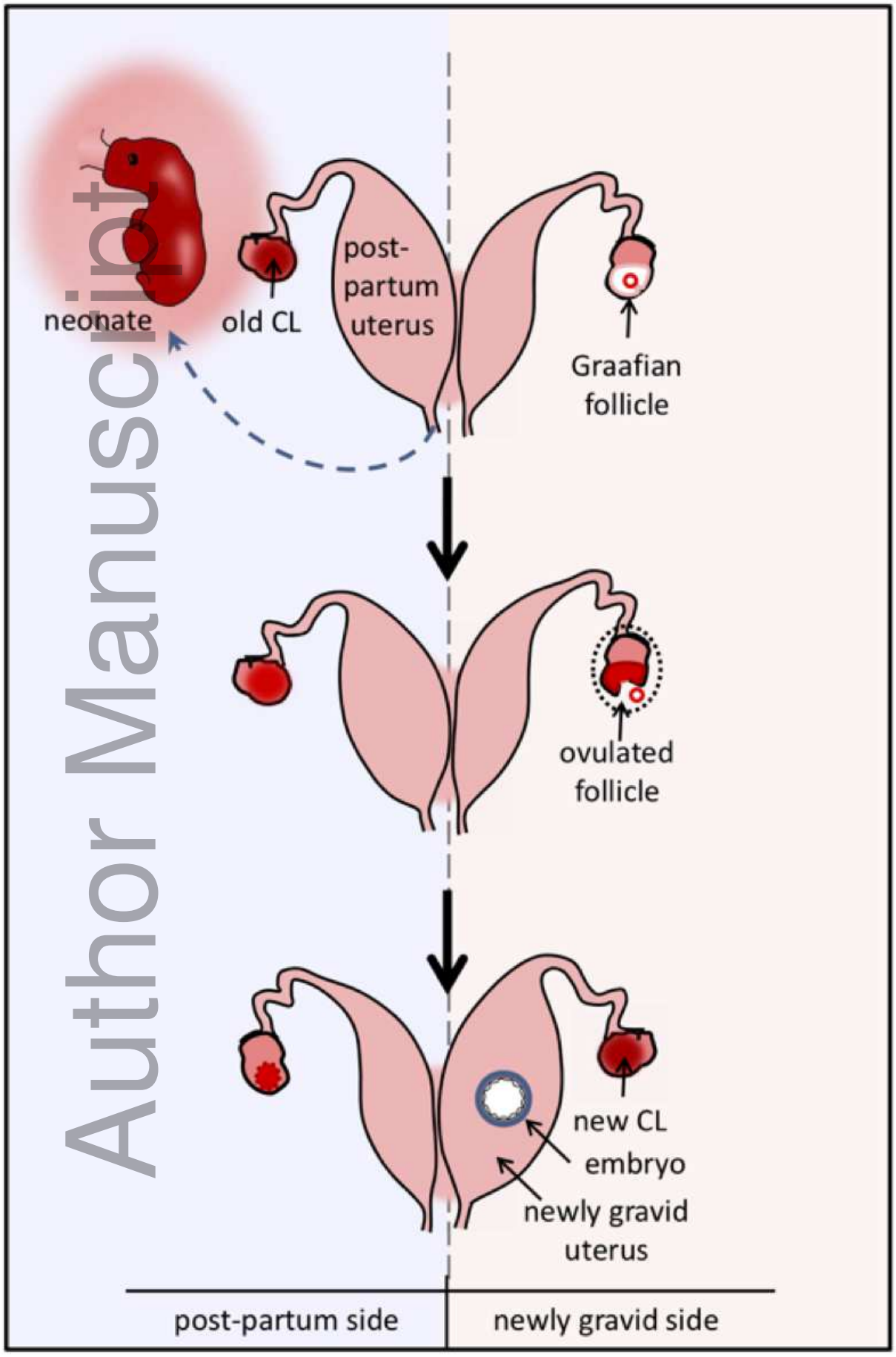
Figure 5: Transmission electron micrographs of glandular epithelial cells of the newly gravid endometrium. Scale bar = 5 μm. A) Day 0 post-partum; B) Day 1-2 post-partum; C) Day 3-5 post-partum; D) Day 6-8. post-partum; E) Diapause; F) Higher magnification of E); Scale bar = 1 μm. Basement membrane (bm), Golgi complex (G), lipid droplets (L), glandular lumen (Lu), lysosomes (Ly), mitochondria (M), microvilli (mv), nucleus (N), rough endoplasmic reticulum (RER), secretory material (s), smooth endoplasmic reticulum (SER), secretory vesicles (SV).

Figure 6A-D: Transmission electron micrographs of glandular epithelial cells of the post-partum endometrium. Scale bar = 5 μm. A) Day 0 post-partum; B) Day 1-2 post-partum; C) Day 3 post-partum; D) Day 5 post-partum. Scale bar = 1 μm. Degenerative cells (deg), Golgi complex (G), intercellular space (ICS), glandular lumen (Lu), lysosomes (Ly), mitochondria (M), nuclei (N), rough endoplasmic reticulum (RER), secretory vesicles (Sv).

Figure 6E-H. E) Day 6-8 post-partum; F) Mid diapause; G) Late diapause; H) Higher magnification of G); Scale bar = 1 μm. Lipid droplets (LD), glandular lumen (Lu), Lysosomes (Ly), elongated mitochondria (M), smooth endoplasmic reticulum (SER), secretory vesicles (Sv).

Figure 7: Transmission electron micrographs of luminal epithelial cells of the newly gravid endometrium. Scale bar = 4 μm. A) Day 0 post-partum; B) Day 1 post-partum; C) Day 3-5 post-partum; D) Day 6-8 post-partum; E) Diapause; Scale bar = 1 μm. Basement membrane (bm), cilia (Ci), Golgi complex (G), glycogen granules (gly), dark granules (gr), lipid droplets (L), uterine lumen (Lu), lysosomes (Ly), basal mitochondria (M), microvilli (mv), Nuclei (N), smooth endoplasmic reticulum (SER), secretory vesicles (Sv), active exocytosis of secretory granules (→).

Figure 8: Transmission electron micrographs of luminal epithelial cells of the post-partum endometrium; Scale bar = 4 μm. A) Day 0 post-partum; B) Day 1 post-partum; C) Day 3-5 post-partum; D) Day 6-8 post-partum; E) Diapause; Scale bar = 1 μm. Cilia (Ci), Golgi complex (G), glycogen granules (gly), uterine lumen (Lu), lysosomes (Ly), mitochondria (M), microvilli (mv), nuclei (N), nucleoli (nu), smooth endoplasmic reticulum (SER), secretory vesicles (Sv).



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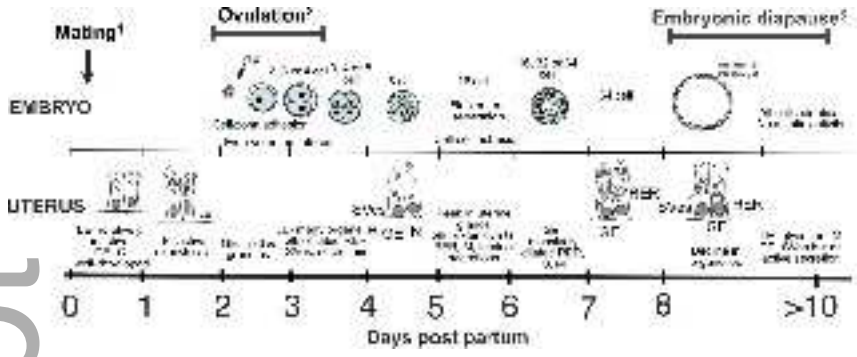

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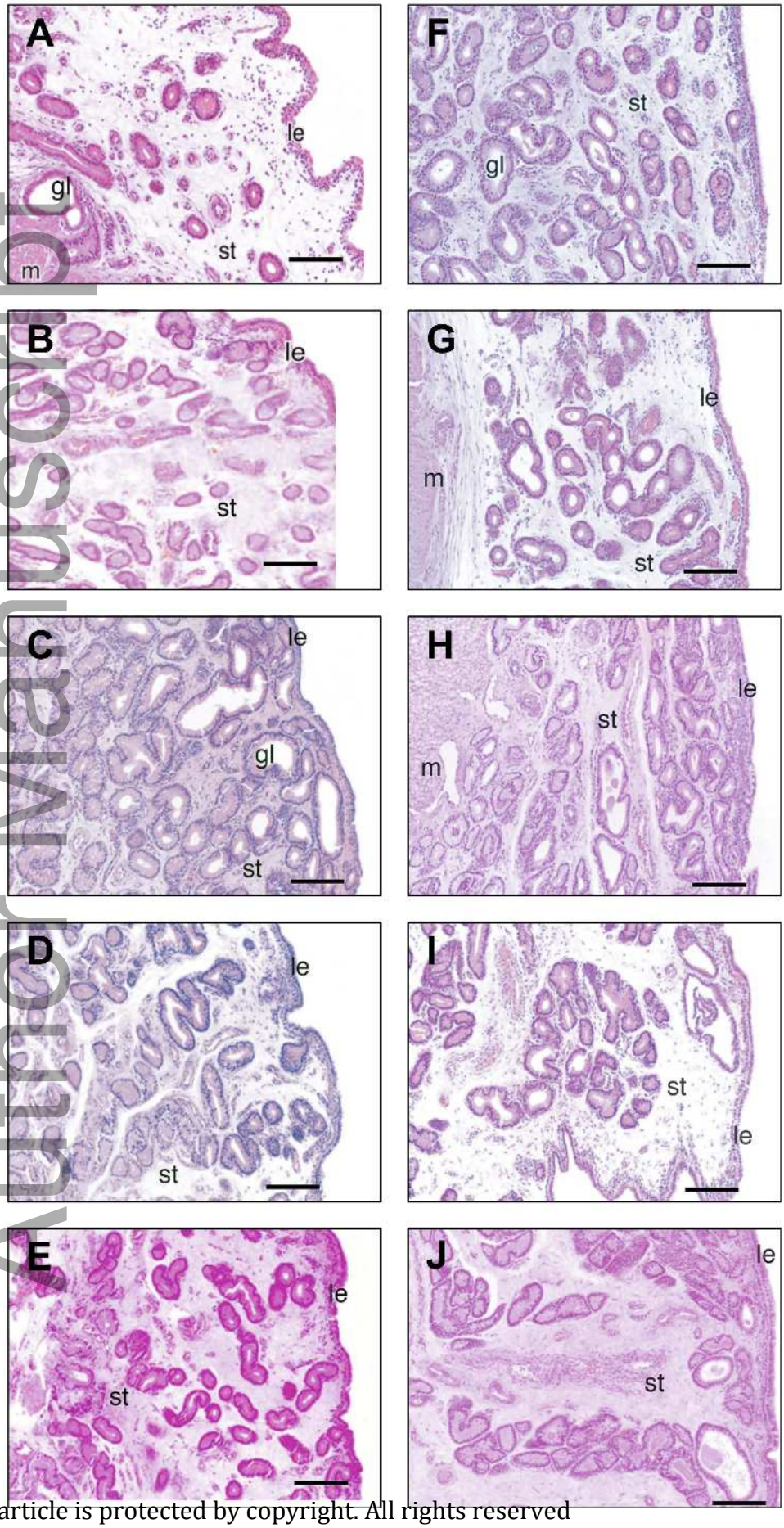

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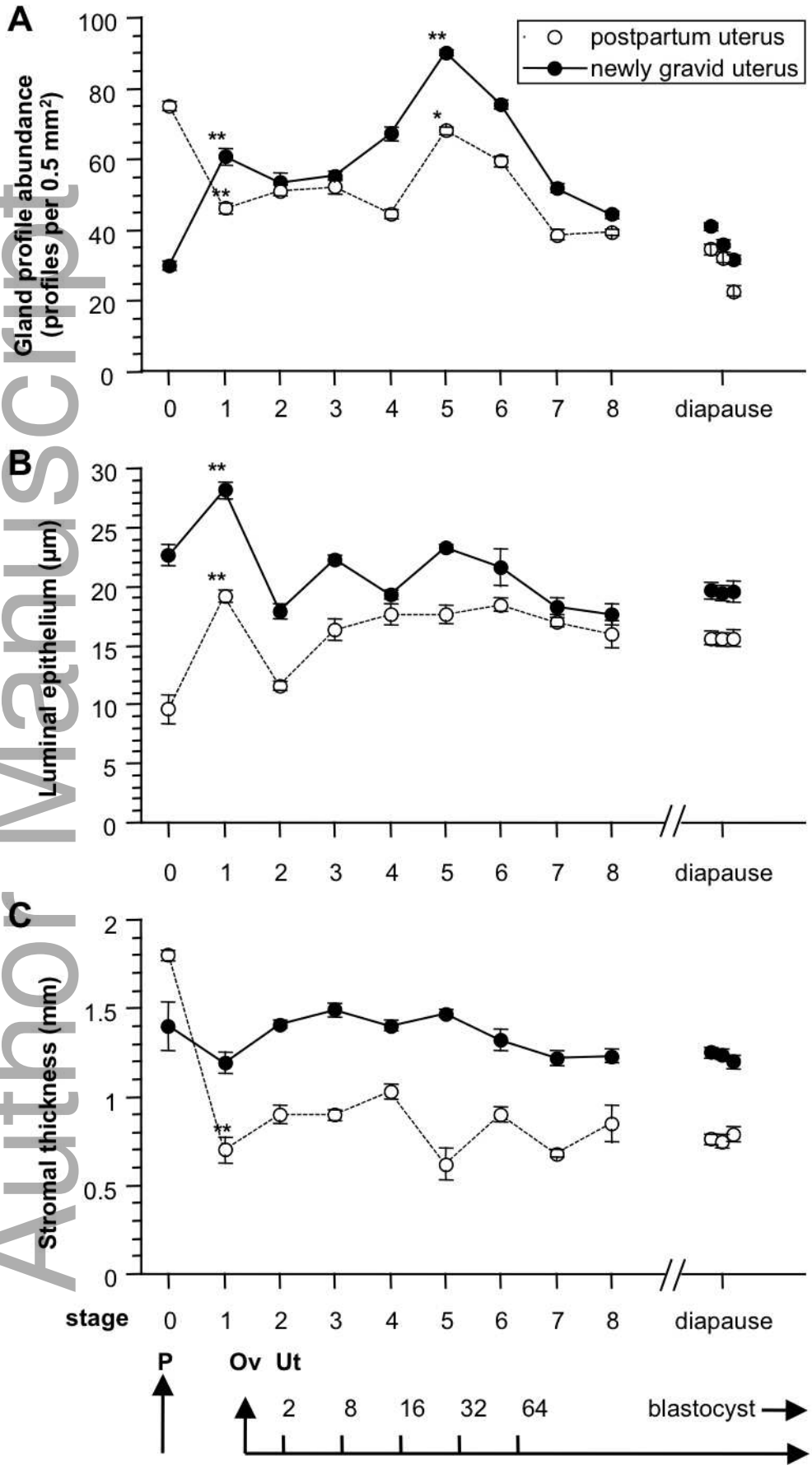

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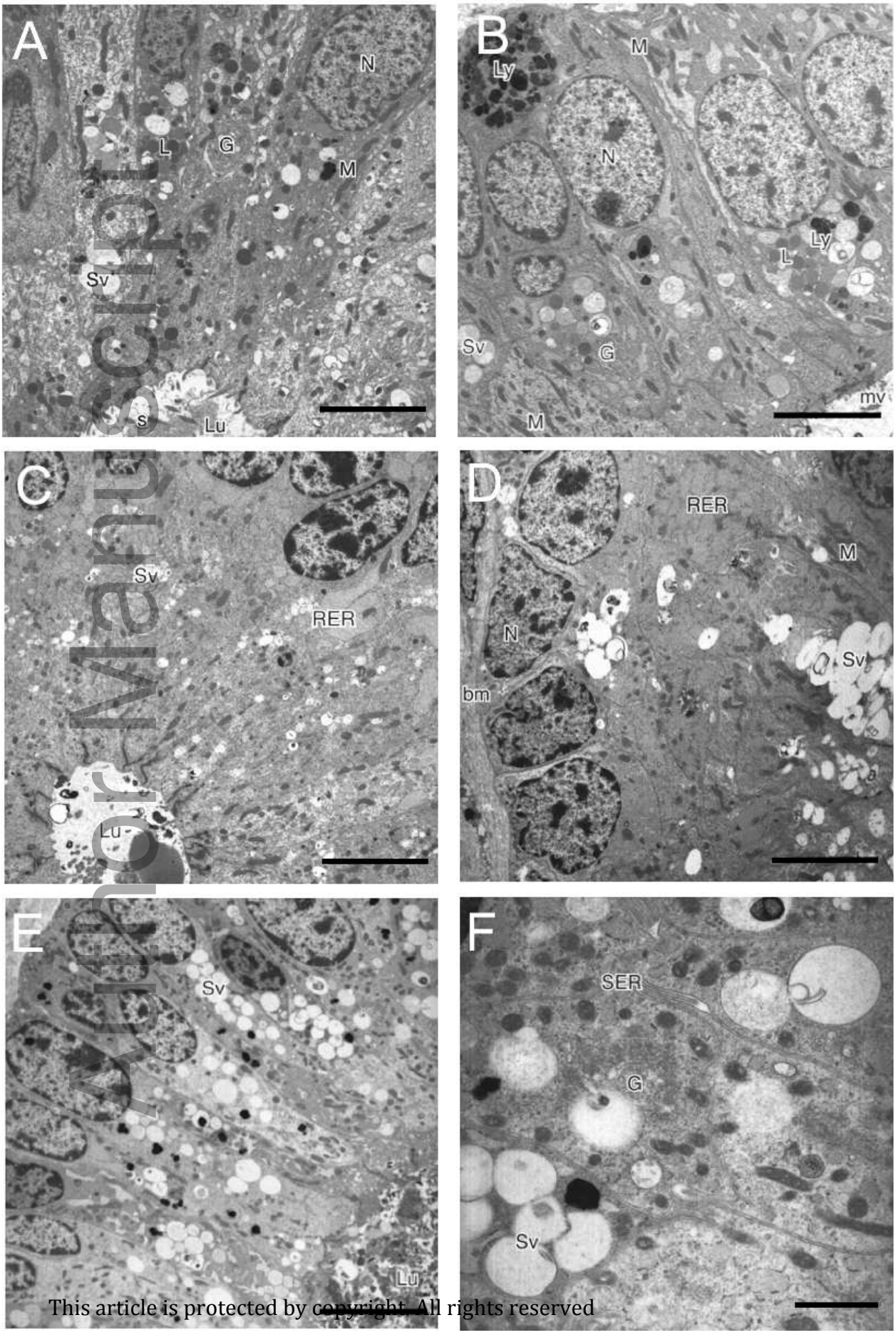

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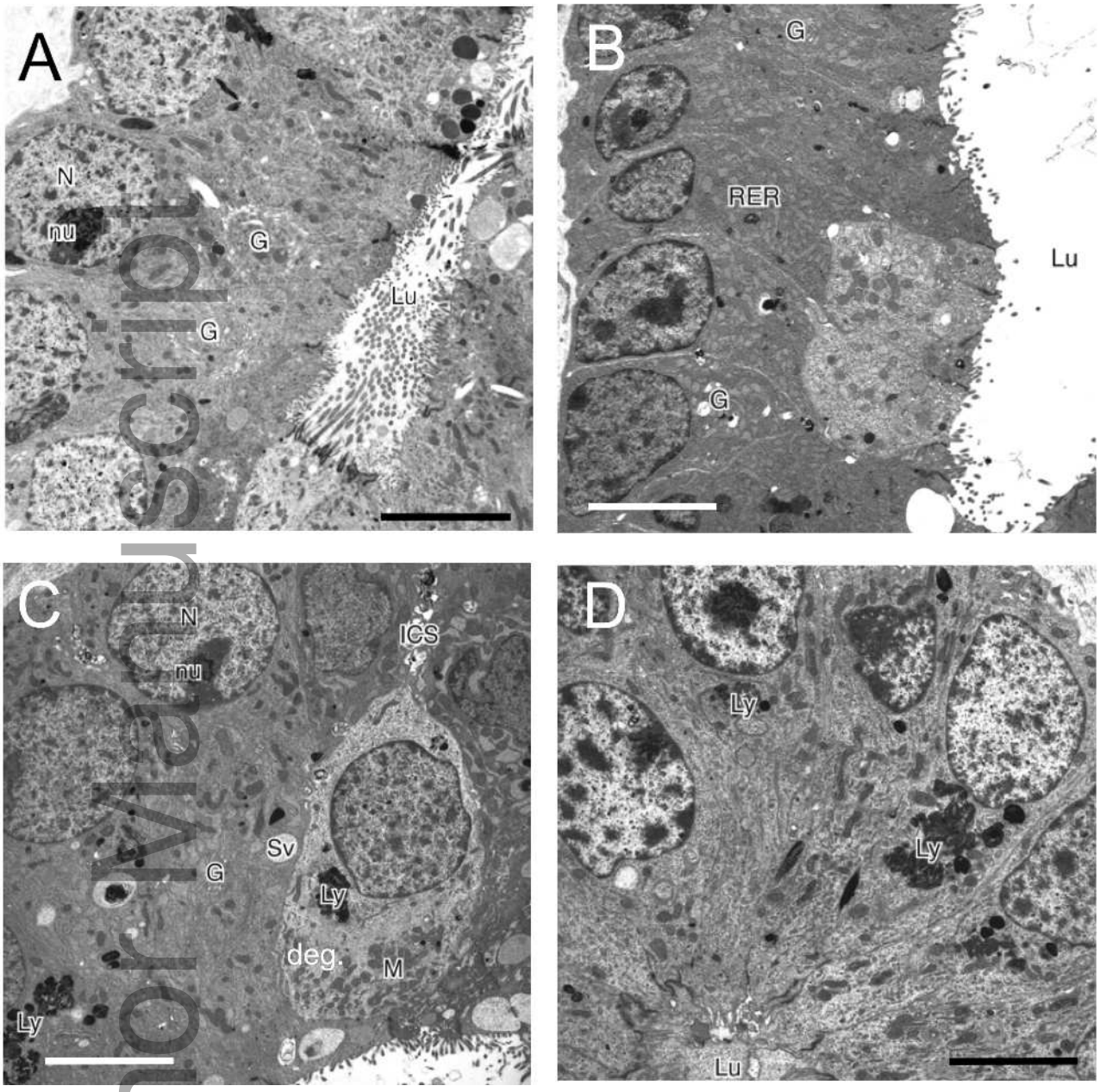

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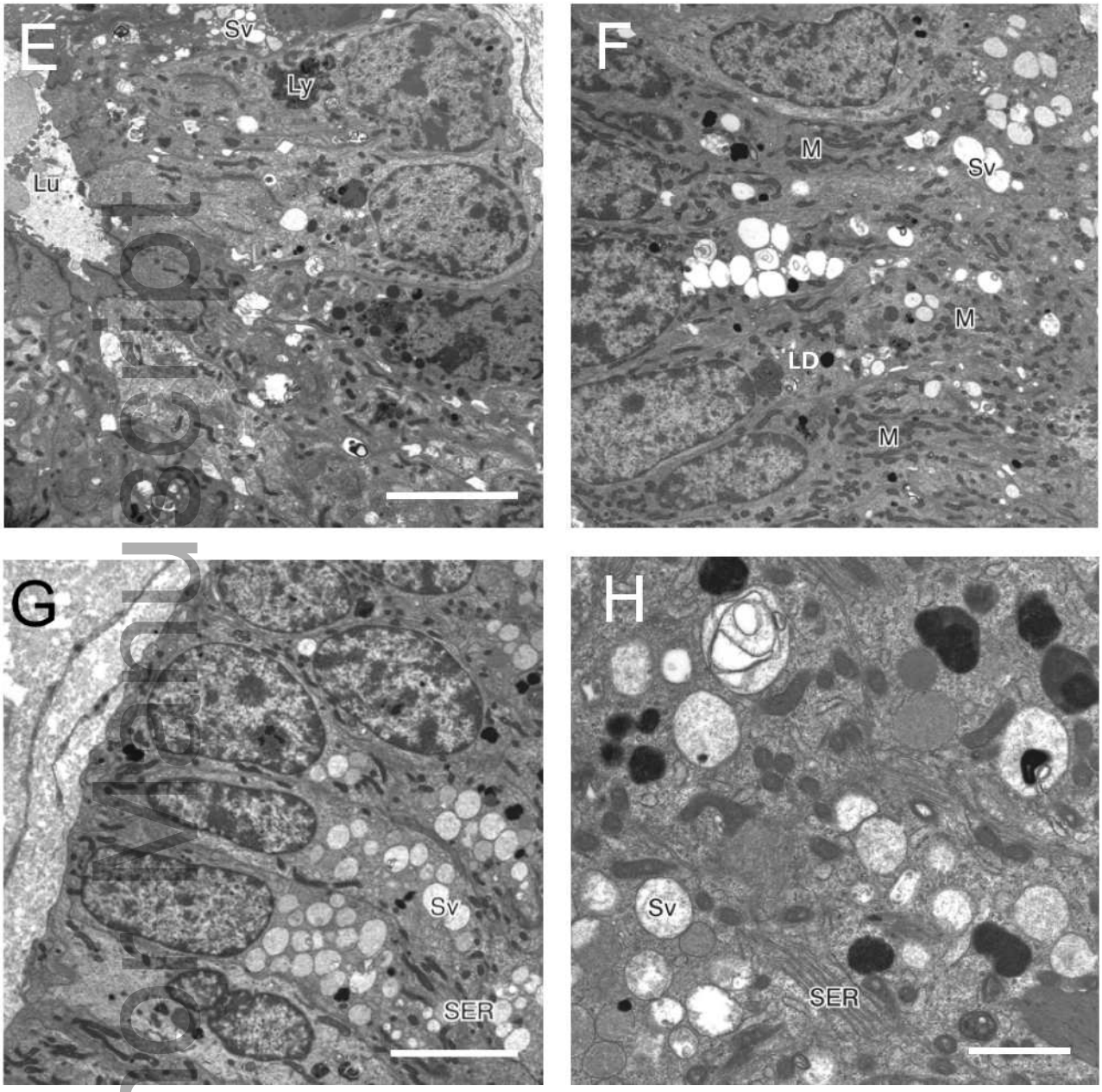

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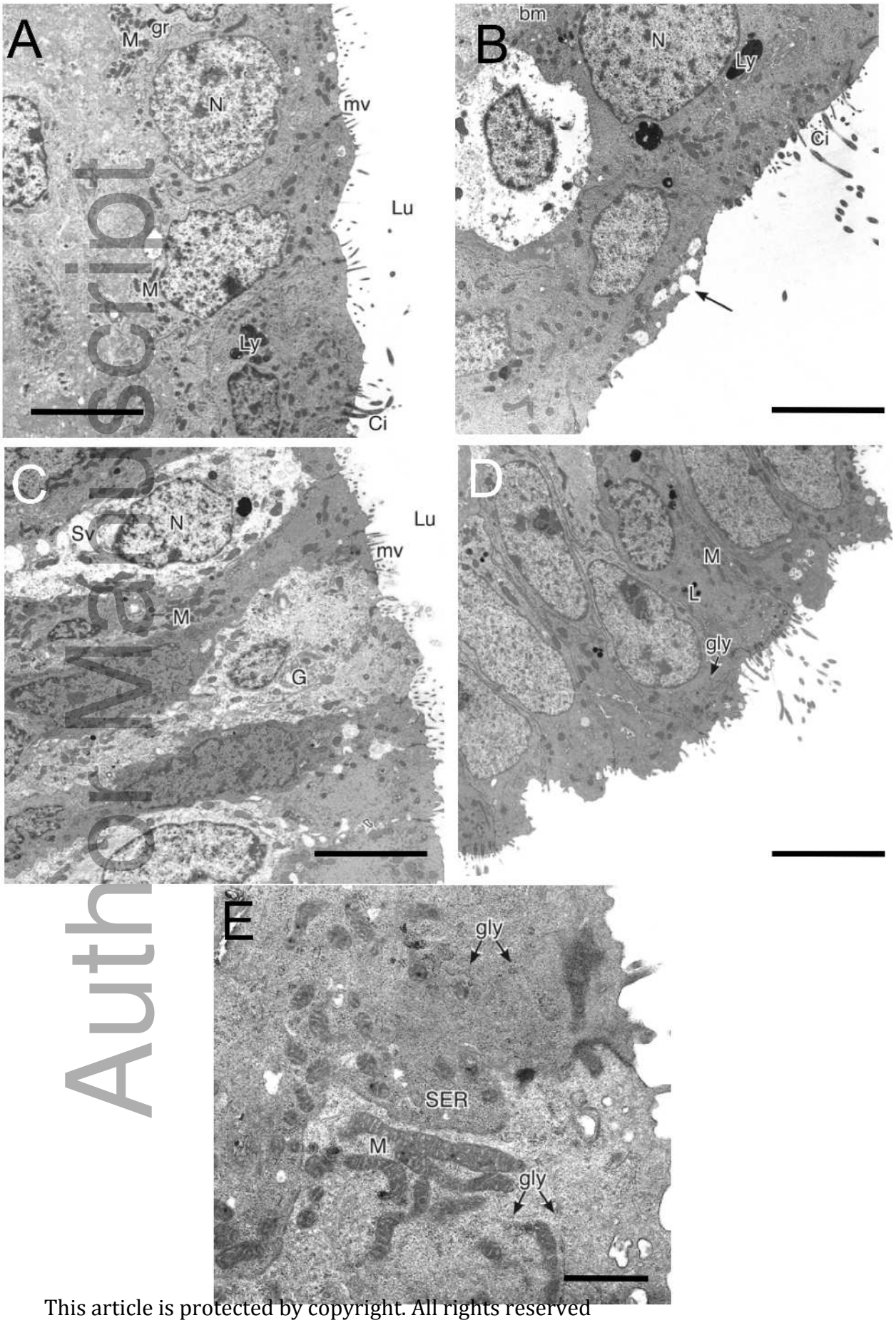

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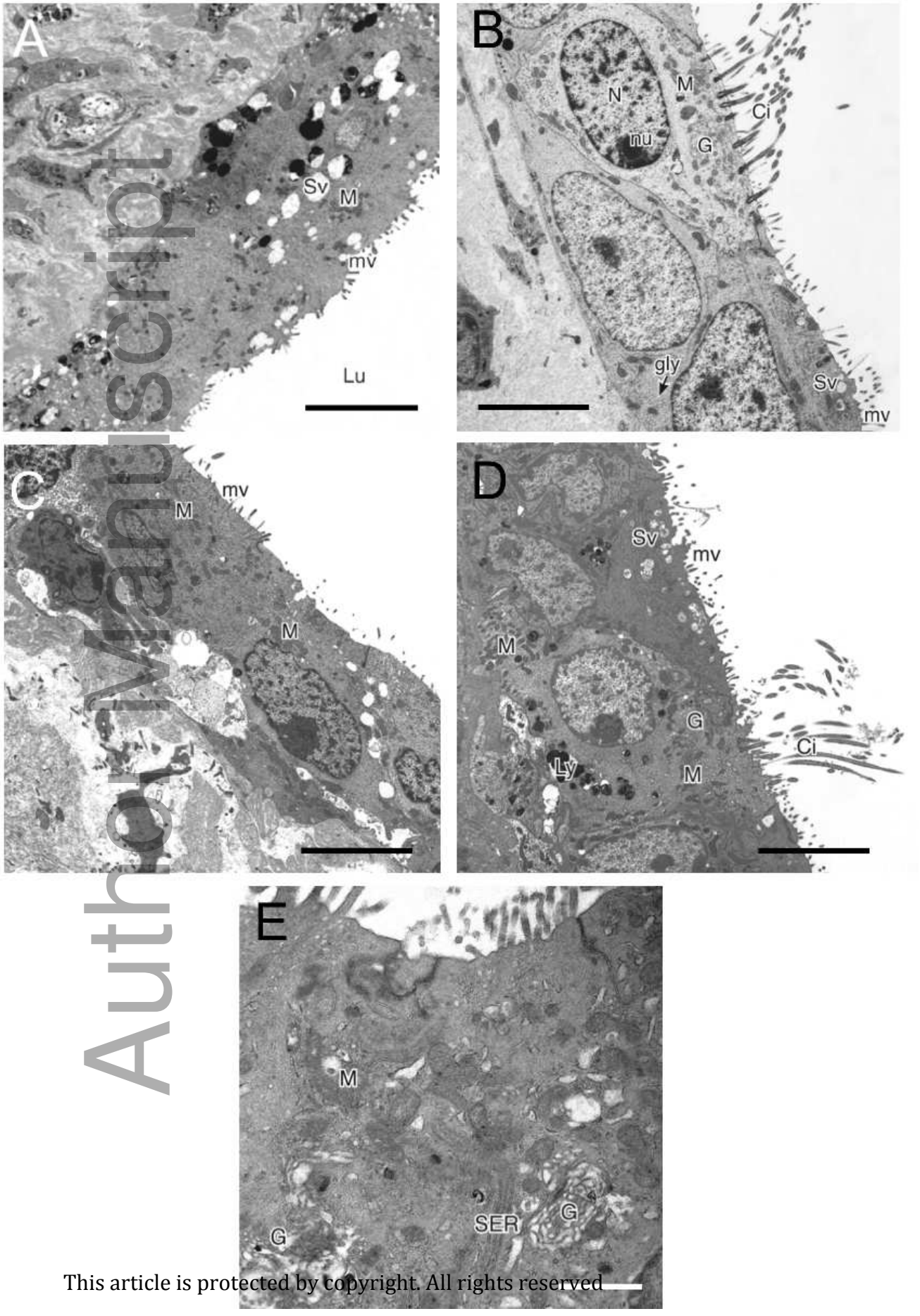

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