Measurement of ATP in Single Oocytes: Impact of Maturation and Cumulus Cells on Levels and Consumption

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Mitochondria provide the primary source of ATP in the oocyte and early embryo and mitochondrial dysfunction and deficit of mitochondria-derived ATP has been linked to suboptimal developmental competence. We have undertaken a study of ATP in the maturing mouse oocyte using a novel recombinant FRET based probe, AT1.03. We show that AT1.03 can be successfully used to monitor cytosolic ATP levels in single live oocytes over extended time periods. We find that ATP levels undergo dynamic changes associated with specific maturational events and that oocytes display altered rates of ATP consumption at different stages of maturation. Cumulus enclosed oocytes have a higher ATP level during maturation than denuded oocytes and this can be abolished by inhibition of gap junctional communication between the oocyte and cumulus cells. Our work uses a new approach to shed light on regulation of ATP levels and ATP consumption during oocyte maturation.

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Oocyte maturation involves a series of nuclear and cytoplasmic changes which result in the formation of an egg which is competent to undergo fertilization. The oocyte resumes the first meiotic division and progresses from germinal vesicle (GV) stage through maturation before arresting metaphase of meiosis II (MII). During oocyte maturation and throughout early development until the blastocyst stage, mitochondria provide the primary source of ATP as glycolysis is limited (Saito et al., 1994; Cetica et al., 2002; Harris et al., 2007). Mitochondria are among the most abundant organelles in the oocyte (Sathananthan and Trounson, 2000) and mitochondrial function is thought to be important for successful maturation and early development. Mitochondrial dysfunction and a deficit in mitochondria-derived ATP in the oocyte have been linked to compromised meiotic maturation and decreased developmental competence (Van Blerkom et al., 1995; Stojkovic et al., 2001; Thoas et al., 2004; Van Blerkom, 2004; Thouas et al., 2006; Zhang et al., 2006; Zeng et al., 2007; Dumollard et al., 2007b; Wang et al., 2009).

In the mammalian ovary, the oocyte is surrounded by cumulus cells that are linked to the oocyte by gap junctions (Anderson and Albertini, 1976). These allow bidirectional communication between the oocyte and cumulus cells, which is necessary for the development and activity of both cell types (Eppig, 2001; Sugiura et al., 2005; Gilchrist et al., 2008). While the mitochondria are the main generators of ATP during oocyte maturation, the oocyte can derive metabolic support from the highly glycolytic cumulus cells which surround it, through the provision of metabolic substrates (Bigger et al., 1967; Donahue and Stern, 1968; Leese and Barton, 1985; Downs and Utecht, 1999). In turn the oocyte is able to influence glycolytic activity and gene expression in the cumulus cells (Sugiura et al., 2007, 2005).

While the importance of mitochondrial function to successful maturation and early development is becoming clear, a detailed examination of ATP supply and demand during oocyte maturation has not been undertaken. Furthermore, although bidirectional communication between the oocyte and cumulus cells can influence oocyte metabolism, the impact on ATP levels in the maturing oocyte is unknown. In light of the correlation between ATP and successful development, we have investigated ATP levels and rates of ATP consumption during mouse oocyte maturation, and examined the impact of cumulus-oocyte coupling on ATP levels in the maturing oocyte. In contrast to previous studies investigating ATP levels in oocyte and embryos which have largely used luciferase assays, we have employed a recently developed recombinant FRET probe (Imamura et al., 2009) to investigate ATP levels in the oocyte.

Materials and Methods
Oocyte collection and culture

Germinal vesicle stage oocytes were collected from 4 to 6-week old female MF1 mice that had been administered 7 international units (IU) pregnant mare’s serum gonadotropin (PMSG) (Intervet, Uden, Netherlands). Oocytes were aspirated from the ampulla of the 输卵管 and cultured in maturation medium consisting of 10% follicular fluid (FF) and 90% modified Earle’s balanced salt solution with HEPES (EBSS-H) supplemented with 10 mmol/l glucose, 100 mmol/l NaCl, 5 mmol/l KCl, 2.5 mmol/l CaCl2, 1 mmol/l MgSO4, 1 mmol/l Na2HPO4, 1 mmol/l KH2PO4, 0.01% gentamicin (Sigma, UK), 1 mmol/l sodium pyruvate, 1 mmol/l L-glutamine, 10 mmol/l HEPES and 100 U/ml penicillin/streptomycin. Oocytes were cultured in a humidified atmosphere of 5% CO2 in air at 37°C for 20 h. Normal fertilization was confirmed by the presence of 2 pronuclear heads following injection with 2 μl of a 1:1 mixture of equine chorionic gonadotropin (eCG) and eCG plus hCG (Serono, Italy) 30 min prior to injection. The cumulus cells were removed by touching the oocytes to a glass slide, washing the cumulus cells away with a pipette and aspirating the cumulus cells into the incubator. Denuded oocytes were then cultured in maturation medium consisting of 15% FF and 85% EBSS-H with 10 mmol/l glucose, 1 mmol/l NaCl, 2.5 mmol/l CaCl2, 1 mmol/l MgSO4, 0.01% gentamicin, 1 mmol/l sodium pyruvate, 1 mmol/l L-glutamine, 10 mmol/l HEPES and 100 U/ml penicillin/streptomycin.
Walton, Milton Keynes, UK) by intraperitoneal injection 48 h earlier. Mice were culled by cervical dislocation and ovaries were collected into warmed M2 medium (Sigma–Aldrich, Dorset, UK) maintained at 37˚C. To release the oocytes, ovaries were punctured with a 27-gauge needle; oocytes were collected with a mouth pipette, and placed in drops of M2 under mineral oil (Sigma–Aldrich) to prevent evaporation. Only oocytes with an intact layer of cumulus cells were selected and these were subsequently removed where applicable by repeated pipetting with a narrow bore pipette. GV arrest was maintained where necessary by the addition of 200 μM 3-isobutyl-1-methylxanthine (IBMX) (Sigma–Aldrich) to the medium. Subsequent release from GV arrest was achieved by repeatedly washing oocytes in M2 medium without IBMX.

To recover mature (MII) oocytes, mice were injected intraperitoneonally with 7.5 IU human chorionic gonadotropin (hCG) (Intervet) 46–48 h after PMSG injection. Oviducts were collected from culled mice 12–14 h after hCG administration. Eggs were released into M2 medium by tearing the oviduct with a 27-gauge needle and cumulus cells were removed by the addition of 300 μg/ml hyaluronidase (Sigma–Aldrich). Oocytes were subsequently washed through three drops of M2 without hyaluronidase and maintained in M2 medium under mineral oil at 37˚C.

**In vitro fertilization**

FM1 male mice were culled by cervical dislocation and sperm were released from the epididymis into T6 medium containing 10 mg/ml M 3-isobutyl-1-methylxanthine (IBMX) (Sigma–Aldrich) to the medium. Subsequent release from GV arrest was achieved by repeatedly washing oocytes in M2 medium without IBMX.

In vitro fertilization

MF1 male mice were culled by cervical dislocation and sperm were released from the epididymis into T6 media containing 10 mg/ml bovine serum albumin (BSA) (Sigma–Aldrich) which had been pre-equilibrated to pH 7.6 at 37˚C in an atmosphere of 5% CO2 in air. Following a 20–30 min swim-out period, 100 μl of sperm solution was added to 100 μl T6 medium under mineral oil and placed at 37˚C in an atmosphere of 5% CO2 in air for 2 h to allow capacitation. In order to carry out IVF, the zona was removed from MII oocytes by brief exposure to acidic Tyrode’s solution (Sigma–Aldrich) at 37˚C followed by repeated washing in M2 medium. Fifty microliters of capacitated sperm solution was added to oocytes in 1 ml of M2 medium on the microscope stage. IVF was carried out 14–16 h after hCG administration.

**Microinjection**

Oocytes were pressure injected using a micropipette and micromanipulators mounted on a Leica Axiovert 135 inverted microscope. A brief over compensation of negative capacitance was used to penetrate the plasma membrane and microinjection was performed using a fixed pressure pulse through a pico-pump (World Precision Instruments, Hitchin, UK). Microinjection volume was estimated at 5% by cytoplasmic displacement.

**Live cell time-lapse imaging of ATP**

Live cell imaging of ATP levels was carried out using AT1.03 or AT1.03RK in a pcDNA3.1 vector. mRNA was produced from linearized template DNA using the mMessage mMachine T7 kit (Applied Biosystems, Paisley, UK) and microinjected as described above. Imaging was carried out at 20 × magnification using an Zeiss Axiovert 200 microscope equipped with a CoolSnapHQ cooled CCD camera and an XBO 75 xenon short-arc lamp microscope illuminating system. A CFP/YFP dichroic mirror was used together with a 430/25 excitation filter for CFP, a 470/30 emission filter for CFP, and a 535/30 emission filter for YFP. The FRET ratio was calculated by dividing the YFP signal by the CFP signal, after background correction. Images were acquired at 1 min intervals for short-term experiments and at 15 min intervals for long-term experiments. MetaFluo software was used to control acquisition and oocytes were maintained at 37˚C by the use of a heated stage. Drugs were added at 10–100 μM concentrations.

**Statistical analysis**

Statistical analysis of experiments with two groups was carried out using the unpaired t-test except for comparison of ATP levels pre- and post-fertilization in which a paired t-test was used. Analysis of variance (ANOVA) with the Tukey–Kramer post-hoc test was used for experiments with three groups. *P*-value < 0.05, **P*-value < 0.01, and ***P*-value < 0.001. Error bars indicate standard error of the mean (SEM) on line graphs. On box and whisker plots, the central line indicates median, the top and bottom of the box indicate 25th and 75th percentiles, and error bars indicate Tukey values.

**Results**

ATP levels have been successfully monitored in live somatic cells using the recombinant FRET probe AT1.03 (Imamura et al., 2009; Bermejo et al., 2010; Liemburg-Apers et al., 2011; Ando et al., 2012; Khodorov et al., 2012; Kishikawa et al., 2012; Surin et al., 2013) but its use in oocytes has not been described previously. We therefore set out to determine if it could be used to measure ATP levels in single live oocytes in real time. We first investigated if decreases in ATP could be detected using AT1.03 by applying oligomycin to inhibit the mitochondrial ATP synthase, which should result in ATP depletion. We conducted time-lapse imaging of oocytes which had been microinjected with mRNA encoding AT1.03 and observed a rapid decline in ATP (Fig. 1Ai), in accordance with results obtained using alternative approaches for monitoring ATP levels (Dumollard et al., 2004). This result was confirmed by application of the protonophore FCCP (Fig. 1Ai). Conversely, no change in FRET signal was observed on application of DMSO as a vehicle control (Fig. 1Ai), or on application of FCCP to oocytes expressing a mutant version of the probe, AT1.03RK (Imamura et al., 2009), which is unable to bind ATP (Fig. 1Ai), indicating that the changes in FRET signal represent changes in cytoplasmic ATP concentration.

We next investigated whether the probe could detect increasing ATP levels in oocytes. This was achieved by first depleting ATP levels by incubating oocytes in substrate free media for 2 h prior to the commencement of imaging (Dumollard et al., 2007a). Pyruvate, the main substrate used by oocytes to generate ATP (Johnson et al., 2007), was then applied and the FRET ratio was monitored. Addition of pyruvate resulted in a rapid increase in FRET ratio, indicating ATP levels were recovering (Fig. 1Bi). In contrast, the FRET ratio continued to decline on addition of a vehicle control (Fig. 1Bi).

Finally, we used the probe to monitor known physiological changes in ATP levels at fertilization. In accordance with previous data (Dumollard et al., 2004; Campbell and Swann, 2006), oocytes expressing AT1.03 showed an increase in ATP levels at fertilization (Fig. 2A,C,E). These changes in FRET ratio were not seen in oocytes expressing AT1.03RK (Fig. 2B,D,E). Together these results indicate that the probe is responding rapidly and specifically to changes in ATP levels in oocytes.

We next set out to examine ATP levels throughout oocyte maturation, a period of 12–14 h. Oocytes expressing AT1.03 were imaged every 15 min for up to 15 h and the rate of polar body extrusion was 73% indicating that oocytes could be imaged for these time periods without deleterious effects on oocyte maturation. We found that ATP levels rose as meiosis I was resumed then decreased approximately 1 h after germinal vesicle breakdown (GVBD). Following GVBD, ATP levels rose gradually before exhibiting a peak during extrusion of the first polar body (PBE). This peak was associated with PBE as it was absent in all oocytes that failed to extrude a polar body (Fig. 3A). Following PBE, ATP levels remained largely stable for...
the remainder of the imaging time. Oocytes expressing AT1.03RK did not exhibit any changes in FRET level (Fig. 3B), indicating that the results obtained with AT1.03 were indicative of genuine changes in ATP level and did not result from changes in cell shape or position. When oocytes were imaged in the presence of IBMX to maintain meiotic arrest during the period of imaging, a modest increase in ATP levels was observed during the initial period of imaging (Fig. 3C). However this was markedly different to the abrupt changes in ATP observed in maturing oocytes, indicating that these are specifically associated with progression of oocyte maturation.

Monitoring ATP during maturation allowed us to measure the steady state level of ATP during this period. However it did not allow us to understand if there are any changes in rates of ATP consumption at specific maturational stages. It has been previously shown that consumption of pyruvate is higher in oocytes undergoing maturation than in oocytes arrested at either GV or MII stage (Downs et al., 2002) indicating that rates of energy consumption may be altered at different stages of maturation. We therefore next investigated the rate of ATP consumption at GV stage, during MI (4–8 h after release from meiotic arrest) and at MII stage. We inhibited ATP synthesis with oligomycin and monitored the rate of ATP decline (Fig. 4A). Using this approach, and analyzing the linear portion of ATP decline, we found that ATP was consumed faster by oocytes undergoing maturation (MI) and at MII stage than in oocytes which were arrested at GV stage (Fig. 4B). To confirm that this did not result from altered levels of glycolysis at different stages, iodoacetic acid was added after oligomycin (Fig. 4A). No further change in ATP level was observed. Nor was any change in ATP level observed when iodoacetic acid was added alone (Fig. 4C), indicating that glycolysis has no impact on ATP levels during oocyte maturation and that the altered rates of ATP decline were a result of different rates of ATP consumption.

Cumulus cells can deliver metabolic support to oocytes by supplying substrates through gap junctions (Donahue and Stern, 1968a,b; Leese and Barton, 1985; Downs and Utecht, 1999; Sugiura et al., 2005). To examine the impact of cumulus cells on oocyte ATP levels we compared the ATP profiles during maturation of denuded and cumulus enclosed oocytes. ATP levels were simultaneously measured in cumulus enclosed oocytes (CEO) and denuded oocytes (DO) during maturation. The pattern of ATP level in CEO was unchanged
when compared to DO, however CEO exhibited higher overall ATP levels than their denuded counterparts (Fig. 5Ai). The ATP level in the early stages of maturation was similar in CEO and DO and there was no significant difference in the level of the peak prior to GVBD (Fig. 5Bi). However, ATP levels in DO subsequently underwent a larger decrease than in CEO after GVBD such that levels in DO were significantly lower than in CEO at this stage (Fig. 5Bii). The lower level of ATP in DO was then maintained throughout the remaining period of maturation such that the level at the end of the imaging period remained significantly higher in CEO (Fig. 5Biii). There was no significant difference in ATP levels between CEO and DO expressing AT1.03RK indicating that the higher ATP levels in CEO were not the result of an imaging artifact (Fig. 5Aii).

Fig. 2. AT1.03 detects physiological changes to ATP level in mouse oocytes. MII oocytes were microinjected with mRNA encoding either (A) AT1.03 (n = 8) or (B) AT1.03RK (n = 3) and capacitated sperm were added as indicated by arrows. Traces shown are the averages of fluorescence ratios measured in each group. C: There is a significant increase in FRET ratio in oocytes expressing AT1.03 but (D) not in oocytes expressing AT1.03RK. E: Example images from oocytes expressing AT1.03 or AT1.03RK. The FRET ratio increases with AT1.03 as ATP levels rise. Warmer colors indicate high FRET activity and cooler colors indicate low FRET activity. Note that although fluorescent intensity increases in both examples as more probe is expressed no change to the FRET ratio is observed with AT1.03RK.
Cumulus cells are believed to provide metabolic support to the oocyte via gap junctions (Anderson and Albertini, 1976; Downs, 1995; Downs and Utecht, 1999). We therefore next set out to determine if gap junctional coupling between oocyte and cumulus cells was contributing to the higher level of ATP in CEO. Gap junctional communication was inhibited using 18α-glycyrrhetinic acid (AGA) and ATP levels in DO and CEO...
during maturation were compared. Oocytes matured normally in the presence of AGA and dynamic changes to ATP level were maintained (Fig. 6A). However, the higher level of ATP observed in CEO matured under control conditions was completely abolished and there was no significant difference in ATP level in CEO and DO in the presence of AGA at any stage of maturation (Fig. 6B). Together these results indicate that cumulus intact oocytes are able to maintain a higher level of ATP during oocyte maturation, likely as a result of metabolic support provided by the cumulus cells through gap junctional communication.

Discussion

Our results reveal interesting new information about regulation of ATP levels and rates of ATP consumption during oocyte maturation and indicate that a new FRET based probe can be used for measuring ATP in live oocytes. Using AT1.03 in oocytes for the first time, we find dynamic changes in ATP levels approximately 1 h after GVBD and during PBE and show that rates of ATP consumption are higher in oocytes at MI and MII than at GV arrest. Finally, we reveal that cumulus enclosed oocytes have higher levels of ATP during oocyte maturation than denuded oocytes, due to metabolic support provided by cumulus cells through gap junctions between cumulus cells and oocytes.

Validation of AT1.03 for use in oocytes

Previous measurements of ATP in oocytes have been conducted using either a destructive luciferase assay allowing measurement of ATP at single time points in a lysate from a population of oocytes, or in single living oocytes by microinjection of luciferase protein or mRNA. Here, we have employed a novel method of measuring ATP in oocytes, using the FRET probe AT1.03 (Imamura et al., 2009). The probe responds rapidly and specifically to changes in ATP in oocytes and persists stably in the oocyte, allowing imaging over long time periods in single live oocytes on a standard fluorescence microscope.

This fluorescence approach to measuring ATP provides a number of advantages over the luciferase technique. Firstly, luciferase protein does not persist for long enough after microinjection to allow long-term imaging, while injection of mRNA results in artifacts from production and destruction of the protein which must be corrected for (Yu et al., 2010). Conventional imaging techniques suffice for measuring ATP using AT1.03, while high end photon counting detectors are needed for measuring luminescence. Furthermore, the development of AT1.03 for measuring ATP raises the possibility of being able to detect subcellular heterogeneity in cytosolic ATP level (Ando et al., 2012), although we did not observe this during the course of our work, suggesting that...
ATP diffuses rapidly in the oocyte, or is highly buffered. One advantage of luciferase is that photons are detected as a result of a chemi-luminescent reaction and therefore measurements are made without the need to expose the cells to excitation light. For photosensitive samples luciferase assays may be more suitable, although the levels of excitation needed for AT1.03 have not proved to be incompatible with high rates of oocyte maturation.

ATP supply and demand during oocyte maturation

The importance of mitochondrial ATP production for energy provision in the oocyte and early embryo is now well established. Glycolytic rates are low in the oocyte and ATP is supplied in the main by mitochondrial oxidative phosphorylation of pyruvate (Biggers et al., 1967; Eppig, 1976; Leese and Barton, 1984; Johnson et al., 2007). At fertilization, mitochondria-derived ATP is essential for sustaining calcium oscillations, which in turn up-regulate mitochondrial ATP production, matching the increased energy demands of fertilization with an increase in ATP supply (Dumollard et al., 2004; Campbell and Swann, 2006). Our data showing the prompt onset of ATP decline on addition of either FCCP or oligomycin is consistent with evidence that mitochondrial oxidative phosphorylation provides the primary source of ATP to the oocyte, and is supported by the finding that inhibition of glycolysis with iodoacetic acid has no effect on ATP levels. The rapid decline in ATP suggests that consumption rates are high in the oocyte which, together with the fact that ATP provision is dependent on mitochondrial oxidative phosphorylation, indicates the importance of mitochondrial function during oocyte maturation.

However, while it has been suggested that ATP content of the oocyte may be relevant for establishing developmental competence (Van Blerkom et al., 1995; Stojkovic et al., 2001; Tamassia et al., 2004; Johnson et al., 2007; Zeng et al., 2007; Zeng et al., 2009), and ATP levels have previously been examined either at discrete time points or continuously during maturation in some species (Van Blerkom et al., 1995; Stojkovic et al., 2001; Sun et al., 2001; Brevini et al., 2005; Yu et al., 2010), a detailed examination of ATP supply and consumption during mouse oocyte maturation has not been described. By undertaking time-lapse imaging of oocytes microinjected with AT1.03 we have been able to detect dynamic changes to ATP levels during oocyte maturation. The pattern of steady state ATP levels we describe is largely in accordance with data using a luciferase based approach (Yu et al., 2010). We observed a sharp drop in ATP approximately 1 h after GVBD and a peak of ATP during polar body extrusion. The fall in ATP after GVBD was over a third of that seen on addition of oligomycin or
FCCP, indicating a significant change in ATP concentration in the oocyte at this time. Interestingly, the peak of ATP we observed at polar body extrusion is in line with a similar peak in oxygen consumption observed in bovine embryos during the first embryonic cleavage (Lopes et al., 2010), suggesting that increased supply of ATP may be associated with cleavage divisions in the oocyte and early embryo.

Our comparison of rates of ATP consumption at GV, MI, and MII stages has revealed significant changes to the rate at which ATP is consumed at different maturational stages. ATP consumption was significantly higher in oocytes during MI and at MII than in those arrested at GV, likely reflecting increased energy demand for processes associated with maturational progress. It has been proposed that reorganization of mitochondria towards the oocyte spindle may be necessary to provide ATP for energy consuming processes associated with spindle formation and function (Van Blerkom and Runner, 1984; Eichenlaub-Ritter et al., 2004) and creatine kinase has recently been shown to be associated with the spindle in mouse embryos and was proposed to have a role in ATP supply for spindle formation (Forsey et al., 2013). These findings are in line with our observation of increased ATP consumption in MI and MII oocytes which contain a spindle when compared to oocytes at the GV stage in which no spindle is present. This also correlates with a report that more pyruvate is consumed by oocytes undergoing maturation than those arrested at the GV stage (Downs et al., 2002). However, the functional significance of the changes to both ATP levels and rates of consumption, and the mechanisms regulating them, remain to be determined. Indeed, we had speculated that the level of ATP during oocyte maturation might be an indicator of oocyte health and correlate with the ability to extrude a polar body but the data did not reveal any such relationship.

The impact of cumulus cells on oocyte ATP

The oocyte metabolizes glucose poorly and is dependent upon oxidative phosphorylation of pyruvate to sustain maturation (Biggers et al., 1967; Brinster, 1971; Barbehenn et al., 1974; Eppig, 1976; Leese and Barton, 1984). In contrast, cumulus cells exhibit high glycolytic activity and it is thought that ATP and energy substrates produced in the cumulus cells are supplied to the oocyte via gap junctions (Anderson and Albertini, 1976; Downs, 1995; Downs and Utech, 1999; Johnson et al., 2007). Indeed, isolated cumulus cells produce pyruvate, supporting this notion (Donahue and Stern, 1968a,b; Leece and Barton, 1985). We have shown that this metabolic cooperation between oocyte and cumulus cells results in a higher ATP level in CEO than DO during oocyte maturation. This higher level was abolished in the presence of a gap junction inhibitor indicating that it is a result of direct cumulus cell–oocyte communication through gap junction channels. In line with our finding of higher ATP in cumulus enclosed oocytes, maturation of oocytes from mice deficient in the pyruvate dehydrogenase enzyme can be partially rescued if they are cultured as part of a cumulus–oocyte complex (Johnson et al., 2007).

Interestingly, the difference in ATP level between CEO and DO appears to be established at the time of the decrease in ATP that occurs shortly after GVBD. It may be that cumulus cell derived metabolic support reduces the fall in ATP which occurs at this stage and allows the oocyte to maintain a higher ATP level during the remainder of oocyte maturation. The higher level of ATP in cumulus enclosed oocytes may have implications for continued development since higher ATP content in human and bovine oocytes has been shown to correlate with a greater capacity for continued development (Van Blerkom et al., 1995; Stojkovic et al., 2001).

In summary, we have described the characterization and use of a novel FRET-based recombinant probe for measurement of ATP levels in individual live oocytes. Live time-lapse imaging of maturing oocytes revealed dynamic changes to ATP levels and ATP consumption throughout maturation. Finally, the presence of cumulus cells was found to influence ATP levels in the maturing oocyte, most likely through metabolic support supplied through gap junctions.

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