A spontaneously arising mutation in connexin32 with repeated passage of FRTL-5 cells coincides with increased growth rate and reduced thyroxine release

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ABSTRACT

In this study we examine changes in the cellular properties of FRTL-5 cells as a function of passage number, with particular emphasis on gap junction expression, karyotype, morphology, growth rate and thyroxine (T₄) release. Early passage FRTL-5 follicular cells transfer dye through gap junctions from injected cell(s) to third-order neighboring cells and beyond within their respective follicles and have immuno-detectable connexin32 (Cx32) type gap junctional plaques in their lateral contacting plasma membranes. By contrast, FRTL-5 cells established as monolayers, or as follicles from cultures passed more than 15 times, did not transfer microinjected Lucifer Yellow dye to contiguous neighboring cells and did not express any immuno-detectable rat thyroid specific connexins (Cx43, Cx32 or Cx26). Western blots confirmed that total, membrane and cytosolic Cx32 protein was present only in early pass follicular cultures. To better understand the passage-dependent loss of Cx32 expression, RT-PCR primers were made to the most unique sequences of the rat Cx32 molecule, the cytoplasmic and carboxyl-terminal regions. These primers were used to screen FRTL-5 RNA

from cultures of various passage numbers. The results revealed that later passage cultures had a single base deletion in the middle of the Cx32 cytoplasmic loop region at nucleotide position 378. This base deletion was in the middle position of the codon for amino acid 116, which is normally a CAC (histidine) but read with the frame shift was a CCC (proline). The four amino acids that followed this deletion were also altered with the fourth one becoming UAA, the ochre translation stop codon. This premature stopping of translation resulted in a truncation of 60% of the protein, which included the remaining cytoplasmic loop, third and fourth transmembrane regions and the carboxyl-terminus. The later passage cultures did not produce a carboxyl-terminal RT-PCR product, indicating that the mRNA was also truncated. These regions of the Cx32 molecule contain the sequences and epitopes to which probes and antibodies are directed, and as such alterations of these regions with repeated passage explains reports by others that FRTL-5 cells do not express Cx32, and implies that cultures used for these assessments were passed more than 15 times. To determine if genetic or epigenetic

abnormalities existed in FRTL-5 cells we performed chromosome spreads from various passage cultures. FRTL-5 cells have been reported to be diploid and more recently non-diploid; however, we found them to be fully tetraploid. This tetraploidy appears to be unstable in that later passes are tetraploid plus two or three extra chromosomes. There were no obvious translocations, breaks or large-scale interstitial deletions of any chromosomes in the FRTL-5 cultures tested. As FRTL-5 cells were repeatedly passed their morphology changed. Monolayer areas spread from beneath the follicles, and the follicles became flattened in appearance. These physical changes were coincident with dramatically increased growth rates. Early cultures (passed 3–12 times) divided on average every 49 ± 1 h, whereas later passes (passes 20–25) divided every 28 ± 3 h. To correlate these changes with a measure of thyroid function we assayed T_4 output. Early passage follicular cultures incubated for 6 h with sodium iodide, released on average 5.27 ± 0.33 ng/ml of T_4 /100 follicles. Later passes, or early

passes treated with heptanol to down-regulate Cx32, released an average of 3.84 ± 0.50 ng/ml of T_4 /100 follicles. There was a 27% difference in T_4 release between early follicular cultures, that were coupled by Cx32, and late or down-regulated early follicular cultures, that were uncoupled ($P < 0.0001$). Collectively, the physical changes documented in this study were coincident with the loss of functional Cx32. This implies a relationship between the loss of intercellular communication and changes in morphogenic appearance, growth rate and reduced thyroid function and supports the previously postulated, tumor-suppressor role for Cx32. FRTL-5 cultures from low passage numbers are an excellent model of primary thyroid cells. However, many reports in the literature ascribe features to FRTL-5 cells that are mutually inconsistent. These differences may be resolved in the future by addressing the passage number and the conditional differences of the cultures being studied.

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INTRODUCTION

The FRTL-5 cell line was derived from a Fischer rat primary thyroid culture and found to be similar to primary thyroid cells in their growth properties and thyroid-stimulating hormone (TSH) dependence. Since their commercial introduction by Ambesi-Impimbato, Parks & Coon in 1980 (Ambesi-Impimbato *et al.* 1980), FRTL-5 cells have been the subject of more than 650 published studies. These investigations fall into a dozen or so categories, emphasizing thyroid and TSH receptor function, organification, signal transduction and follicular assembly (Chen *et al.* 1993, Kimura *et al.* 1997, Cavalieri *et al.* 1999, Suzuki *et al.* 1999, Ariga *et al.* 2000).

Normal rat thyroid cells express three well-characterized connexin (Cx) proteins: Cx43, Cx32 and Cx26 (Meda *et al.* 1993, Green *et al.* 1996). Connexin monomers form hexameric rings in the plasma membranes of contacting cells. When two connexin hemi-channels in adjacent cells unite, they form a complete channel or connexon. Through lateral diffusion aggregates of multiple connexons form a gap junction (Unwin & Zampighi 1980). Gap junctions establish cytoplasmic continuity between coupled cells and regulate numerous cellular properties, including growth and differentiation (Loewenstein 1979, Salomon & Meda 1986, Dermietzel 1993, Holder *et al.* 1993, Temme *et al.* 1997). We have previously documented that in

organized rat thyroid follicles the three connexins assemble at different thyrocyte cell surfaces: Cx43 localizes at the basal surface connecting cells in adjacent follicles; Cx32 at the lateral surface unites cells within a follicle; and Cx26 at apical surfaces faces the lumen (Green *et al.* 1996). There is a morphogenic control over the expression of Cx43 and Cx32. Cx43 predominates when the cells are in monolayers, and Cx32 gap junctions assemble as the cells undergo follicularization (Munari-Silem *et al.* 1994, Green *et al.* 1996, 1997). The location of Cx26 suggests that it is transported to the apical surface by vesicular transport from the trans-Golgi complex once the polarity is established by the lateral assembly of tight and adherens junctions (Yap *et al.* 1995).

An important aspect of endocrine/exocrine glands is their polarized assembly, which at the most intimate level includes gap junctions (Meda *et al.* 1993). Cx43, Cx32 and Cx26 are regulated differently (Saez *et al.* 1993), and associate with different thyrocyte cell surfaces (Meda *et al.* 1993, Guerrier *et al.* 1995, Green *et al.* 1995a, 1996) wherein they are expected to contribute to different aspects of thyroid function. The location of Cx32 at the lateral surfaces of follicular thyrocytes establishes metabolic cooperativity between the coupled cells. This allows them to act collectively in their response to TSH/cAMP signaling and subsequent thyroid hormone release. Thus, the histotypic up-regulation of Cx32 with follicularization (Munari-Silem *et al.*

1994) and its lateral location implies that of the connexins, Cx32 is most important to coordination of thyrocytes organized as follicles (Green *et al.* 1995a, 1996).

Gap junctions have been postulated to serve as tumor suppressor proteins, in that, when absent, either physically or functionally, the uncoupled cells have dramatically increased growth rates (Temme *et al.* 1997, Mehta *et al.* 1999, Yamasaki *et al.* 1999a). Cx32 is the predominant connexin in liver and when down-regulated either naturally or artificially (knockout mice), the incidence of hepatocarcinoma is greatly increased (Tsuda *et al.* 1996, Temme *et al.* 1997). When Cx32 was re-introduced by transfection into FRTL-5 cells, that were not expressing Cx32, their growth rates were substantially reduced (Statuto *et al.* 1997).

Individual follicles are discrete functional units of the thyroid gland and the lateral assembly of Cx32 gap junctional channels in part establishes their coordinated function. This is exemplified under conditions of inflammatory injury, where the decreased expression and function of Cx32 contributes to the resulting hypothyroidism. Normally, gap junction-competent thyroid follicles respond to TSH signaling collectively, via their Cx32 junctional channels, and release the required levels of the thyroid hormones. In gap junction-uncoupled thyroid follicles the response to TSH by individual thyrocytes is insufficient to meet the demand and so the levels of TSH remain elevated (Green *et al.* 1995a, 1996, 1997). A similar situation has been documented in beta islet cells of the pancreas. There, the output of insulin by individual cells was significantly less than when they were homotypically coupled by gap junctions (Meda 1989). Thus, for liver, pancreatic islet and thyroid follicles the presence of gap junctions integrates individual cells into functional tissue units, allowing them to regulate their growth and to act collectively in their receipt and response to various signals.

We purchased FRTL-5 cells from the American Type Culture Collection (ATCC) in the early 1990s and maintained them in parallel with our murine (Green *et al.* 1995a,b) and rat (Green *et al.* 1996, 1997) primary thyroid cultures. The cultures from early passages (<15) closely resembled primary thyrocytes: they developed into well-defined follicles in culture; required TSH for growth; divided on average every 49 h; and produced thyroxine (T_4) when supplied with iodide. We continued to carry FRTL-5 cells comparing them to primary rat thyroid cultures, and have compiled information regarding the conditional dependence of their functional properties.

In this report we address phenotypic changes in FRTL-5 cells as a function of passage number, including gap junction expression, karyotype, morphology, growth rate and T_4 release. Additionally, we discuss how some apparently conflicting reports in the literature can be explained by differing conditions and/or passage numbers.

MATERIALS AND METHODS

Fisher rat thyroid cell line/tissue culture

FRTL-5 cells were purchased from the ATCC (Manassas, VA, USA). Upon receipt, the cells were grown expanded and samples were stored frozen in liquid nitrogen. The cells used in these experiments were derived from our pass 3 through pass 25 FRTL-5 stocks. Our care and feeding of these cells has been previously described (see Green *et al.* 2001). For clarification, Ham's modified F-12 is the medium specified by ATCC, we however, maintain our FRTL-5 cells in DMEM:F12 (50:50 v/v). We grew the FRTL-5 cells in both media for comparison prior to switching to the DMEM:F12 mix. There were no differences in the growth or physical properties of the cells grown in the two different media. We therefore opted to use the same medium that we developed for use with our primary rodent thyroid cultures (Green *et al.* 1995a). The calf serum used has been from two pre-tested lots, one purchased in 1993 and more recently a restock in 1998 (Summit Biotechnologies, Ft Collins, CO, USA). When T_4 measurements were to be taken, 1 nM sodium iodide was added to the complete culture medium at least 6 h prior to harvesting supernatant. To test for T_4 release in cultures with down-regulated Cx32 gap junctions, 3 mM heptanol was added to established follicular cultures 1 h prior to the addition of sodium iodide.

Microinjection

The electrophysiological measurements were done as previously described (Green *et al.* 1995a). All observations and microinjections were performed on an Olympus IX-70 optical base (Scientific Instruments, Temecula, CA, USA) equipped with long working distance objectives and a C-mount analog color camera (Oly-750). The camera was connected to an image processing system (ImagePro Plus version 4, Media Cybernetics, Silver Springs, MD, USA) operated by a computer (200 MHz Pentium PC). Fluorescent images were captured and stored as TIFF files.

Fluorescence immunocytochemistry

FRTL-5 cells established on coverslips were fixed as previously described (Green *et al.* 1996, 1997). Primary rabbit polyclonal antisera directed against Cx43 (CT-360) were obtained from Dr Dale Laird (Laird *et al.* 1991) and an additional rabbit polyclonal anti-Cx43 and anti-Cx26 were obtained from Zymed Laboratories, Inc. (San Francisco, CA, USA). Monoclonal antibody against Cx32 was purchased from Chemicon International (Temecula, CA, USA), and a rabbit polyclonal antiserum directed against Cx32 was obtained from Dr Klaus Willecke (Winterhager *et al.* 1991). The secondary antibodies, FITC-conjugated goat anti-rabbit IgG and FITC-conjugated donkey anti-mouse IgG were purchased from Chemicon International.

To reveal the lateral surfaces of organized thyroid follicles, established cultures were gently lifted with collagenase A and centrifuged (1000 r.p.m., 7 min). The pellet was covered in cryoprotectant and frozen. The frozen follicles were cryosectioned, fixed and immuno-labeled as described above. Non-specific fluorescence was determined by incubating replicate coverslips with pooled normal, non-immune sera (Sigma, St Louis, MO, USA) and secondary antibody, or with secondary antibody alone. The fluorescent images were captured using ImagePro Plus software via an Oly-750 analog color camera connected to an Olympus BX-60 microscope (Scientific Instruments).

Protein fractionation, immunoprecipitation and Western blot analyses

FRTL-5 cells from various passage numbers were grown to approximately 80% confluence, and cells harvested by scraping in cold immunoprecipitation buffer (IPB; 100 mM $\text{Na}_2\text{H}_2\text{PO}_4$, 150 mM NaCl, 2 mM EDTA, 1 mM sodium orthovanadate, pH 7.0), 1 ml/T-75 flask. The protein cell lysates were stored on ice and further disrupted by shearing with a 22-gauge needle. Aliquots (100 μl) of the sample solutions were saved (total lysate), and the remaining lysate ultra-centrifuged at 100 000 *g* for 1 h at 4 °C to separate cytosolic from membrane protein. The membrane pellets were then re-suspended with IPB buffer and further sheared with a 25- and then a 27-gauge needle. NP-40 (2%, v/v) was added to each sample (total protein lysate, cytosolic, and membrane lysates), followed by the addition of 50 μl of Protein A-agarose (Gibco-BRL, Gaithersburg, MD, USA) for 4 h at 4 °C to pre-clear any non-specific binding to protein A agarose. The clarified supernatants were transferred to clean

micro-centrifuge tubes and quantified by Bio-Rad (Hercules, CA, USA) protein reagent.

To 100 μg protein from the sample cell lysates, 15 μg anti-Cx32 were added and incubated at 4 °C with constant rocking for 16 h. Protein A-agarose beads (100 μl) were added to the samples and incubated for an additional 16 h at 4 °C with constant mixing. Cx32-anti-Cx32 bound agarose beads were collected by centrifugation at 12 000 *g* for 30 s and washed three times with immuno-precipitation wash buffer (0.5% Tween-20, 50 mM Tris pH 7.5, 150 mM NaCl, 0.1 mM EDTA) and once with triple-distilled H_2O . Cx32-Protein A complex was then re-suspended in 30 μl lysis buffer (IPB supplemented with 150 mM β -mercaptoethanol, 5 mM dithiothreitol, 1% Triton X-100 and 3% SDS) and incubated for 30 min at room temperature to release Cx32 protein from the antibody and Protein A beads.

Western blot procedures have been previously published by Green *et al.* (1997). Briefly, 15 μg of the immunoprecipitated protein samples were mixed with an equal volume of twofold concentrated sample buffer and 30 μl were loaded, without prior heating, onto a 10% Tris-glycine gel (Novex, San Diego, CA, USA). Pre-stained molecular weight markers (Bio-Rad) were denatured at 65 °C for 5 min and electrophoresed in parallel on each gel. The membranes were hybridized with 3 $\mu\text{g}/\text{ml}$ primary antibody overnight at 4 °C, washed and incubated with 5 $\mu\text{g}/\text{ml}$ secondary antibody (Cy-5-conjugated donkey anti-mouse IgG) in blocking buffer for 4 h at room temperature. The resulting blots were scanned to TIFF files using a Molecular Dynamics fluorescence analysis system, STORM model 860 (Molecular Dynamics, Sunnyvale, CA, USA) equipped with ImageQuant software.

Molecular biology

Northern blot analysis

RNA isolation, gels and hybridization protocols used have been previously published (Green *et al.* 1995a). FRTL-5 cells, established in flasks, were rinsed in serum free medium and 1 ml/T-75 flasks (approximately 5×10^6 cells) of guanidine thiocyanate were added. The RNA was extracted from the cell lysates with phenol followed by alcohol precipitation and quantified by spectrophotometry (A_{260}/A_{280}). Isolated cDNAs were labeled with ^{32}P -dCTP using a random priming technique (Feinberg & Vogelstein 1983). Agarose gels containing approximately 20 μg total RNA were electrophoresed, transferred to Hybond-N membranes, u.v. cross-linked, stained with methylene blue and the ribosomal bands marked and photographed.

TABLE 1. Dye transfer in FRTL-5 cells

	Degree of transfer ^a			<i>n</i> ^b
	1°	2°	≥ 3°	
<i>Early</i> ^c				
Monolayer ^d	2 ^e	1	0	125
Follicle ^f	98	90	75	250
<i>Late</i> ^g				
Monolayer	1	0	0	110
Follicle	1	1	0	200

^aThe distance of Lucifer Yellow dye transfer from the injected cell to concentric, contacting neighboring cells, measured by degree. Primary transfer (1°), secondary transfer (2°), tertiary (3°) and beyond.

^bThe number of trial injections.

^cFRTL-5 cells passed from 5 to 12 times.

^dCultures dispersed and grown as a single cell layer.

^ePercent of cell to which dye is transferred.

^fCultures grown as organized follicles.

^gFRTL-5 cells passed from 16 to 25 times.

The blots were hybridized with the cDNA probes at 42 °C for 36 h, washed (twice in 6 × NaCl, NaPO₄ EDTA (SSPE) at 42 °C for 15 min/wash), and placed on Fugix-ray film (Fisher Scientific, Tustin, CA, USA) for 72 h. The resultant films were scanned and printed.

RT-PCR

RNA samples isolated from various passage FRTL-5 cultures were tested for expression of rat Cx32 message using the Stratagene ProSTAR HF Single-Tube RT-PCR System (Stratagene, La Jolla, CA, USA), which combines cDNA synthesis and high-fidelity PCR amplification in a one-step format. Primers specific for the cytoplasmic loop (nucleotide position 343–424) and C-terminus (nucleotide position 705–895) of rat Cx32 were designed using Do Primer (www.DoPrimer.com) (17–19 base pairs (bp) in length), and synthesized in the Center for Molecular Biology and Gene Therapy (Loma Linda University, Loma Linda, CA, USA). The reaction procedure was performed according to the manufacturers' instructions. The mixture contained 39.5 µl RNase-free water, 5 µl 10 × RT-PCR buffer (provided with the kit), 1 µl primer set (sense and nonsense loop or C-terminus, 100 ng/µl), 1 µl dNTP mix (40 mM), 1 µl RNA sample (200 µg/ml), 1 µl MMLV-RT enzyme, and 0.5 µl TaqPlus Precision DNA polymerase. Control vials for the loop and C-terminus received all components except FRTL-5 RNA. The complementary DNA synthesis and PCR took place during 40 cycles of an uninterrupted thermal-cycling program – 15 min of 37 °C incubation for cDNA synthesis followed by a denaturing step of 1 h at 95 °C, a template-primer annealing step of

FIGURE 1. Gap junction-mediated cell-to-cell communication in FRTL-5 cells. The composite is arranged with phase images (a, c and e) to the left of the corresponding fluorescent images (b, d and f). The phase images are shown to indicate the morphology and extent of membrane contact not evident from the fluorescent image alone. Arrowheads indicate the cell that was microinjected with Lucifer Yellow dye. Images were captured within 2 min post injection. Monolayer cells did not transfer dye to neighboring cells (a and b). Early passage FRTL-5 cells (pass 9) transferred dye to contiguous cells within the follicle (c and d). Late passage FRTL-5 cells (pass 21) were severely reduced in their ability to transfer dye (e and f). See Table 1 for values. Magnification is approximately 40 ×.

30 min at 48 °C (cytoplasmic loop) and 52 °C (C-terminus), and an extension step of 2 h at 68 °C. RT-PCR products were then separated on 2% (w/v) low melting temperature agarose gels by electrophoresis at 100 V for 1 h. The gels were then stained with ethidium bromide, and photographed using an AlphaEase Digital Imaging and Analysis System (Alpha Innotech Corporation, San Leandro, CA, USA).

FIGURE 2. Immunocytochemical labeling of Cx32 gap junctions in FRTL-5 follicular cultures. Shown is a representative follicle from pass 9 cultures (panel a) which express a high level of Cx32 plasma membrane assembled gap junctions. In contrast, no punctate immunofluorescence was detected in the pass 20 FRTL-5 cells (panel b). The low level of fluorescence seen in panel b is equivalent to non-specific binding (data not shown). Images captured at $40\times$ magnification.

DNA purification and sequencing

The designated bands from the RT-PCR gels were cut and purified using Qiaex II Gel Extraction Kit (Qiagen, Valencia, CA, USA). The cut bands were melted in a mixture of 400 μ l QX1 buffer and 15 μ l Qiaex II beads at 50 °C for 15 min. After centrifugation at 14 000 r.p.m. for 30 s, the pellets were washed once in QX1 buffer to remove any remaining agarose, then twice to remove residual salt contaminants. After air-drying for 2 h at room temperature, the pellets were eluted in 10 mM Tris and the DNA supernatants were sent to the Center of Molecular Biology and Gene Therapy (Loma Linda University) for sequencing.

BLAST alignment

To determine if the RT-PCR products corresponded to the expected regions, the resulting DNA sequences were aligned with the known rat sequence database from the National Center for Biotechnology Information web site (www.ncbi.nlm.gov), using the BLAST (Basic Local Alignment Search Tool) program.

Karyotyping

Metaphase chromosome spreads from FRTL-5 cells were prepared by first treating the cells with 0.6 μ g/ml colcemid for 24 h at 37 °C in 5% CO₂.

FIGURE 3. Immunophenotyping of connexin expression in FRTL-5 cells. Fixed FRTL-5 follicular cryosections from pass 12 cultures were incubated with anti-Cx43 (panel a), anti-Cx32 (panel b), and anti-Cx26 (panel c) and FITC-conjugated secondary antibody. There was no detectable Cx43 or Cx26, however Cx32 was expressed at a high level located in the lateral contacting surfaces. Magnification is approximately $100\times$.

After the colcemid treatment, the cells were lifted from the flasks by trypsinization and centrifuged (1500 r.p.m., 10 min), washed once with medium and resuspended with continual mixing in 5 ml KCl (0.55 g/100 ml H₂O). The cells were incubated for 30 min in the salt solution (25 °C) and then 3 ml fixative (methanol:acetic acid, 3:1 v/v) were added and the samples left undisturbed for 12 min. When this incubation was complete, the cells were centrifuged and resuspended in 10 ml fixative and left undisturbed for 30 min (Oshimura & Barrett 1986). Spreads were made by the drop-wise application of the samples onto clean, pre-labeled microscope slides. The slides were allowed to dry prior to staining with Giemsa (0.4%) and covered with glass coverslips using Permount (Fisher Scientific).

MTS assay for cell proliferation and growth rate

The growth rate of various passages of FRTL-5 cells was determined using the CellTiter 96 AQ aqueous one solution proliferation assay from Promega (Madison, WI, USA). This colorimetric method determines the number of viable cells by their ability to bio-reduce a novel tetrazolium compound (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2H-tetrazolium, inner salt; MTS) and an electron-coupling reagent (phenazine ethosulfate; PES). PES has enhanced chemical stability, which allows it to be combined with MTS to form a stable, colored formazan, a product soluble in tissue culture medium. Cells were resuspended at 2×10^5 /ml and 100 µl aliquots of 2-fold serially diluted samples (eight replicates per sample) were plated in 96-well microtiter plates. The time course study was conducted over 72 h with samples taken at various time intervals. At the desired time, 20 µl MTS were added to each well and the plate was returned to the incubator. After 3 h, 100 µl supernatant were transferred to a clean 96-well plate(s) and read at 490 nm wavelength in a Bio-Rad microplate spectrophotometer. The quantity of formazan product was measured by the amount of 490 nm absorbance, which was directly proportional to the number of living cells in the culture. The cell growth rates were calculated based on standard curves using the 0 h time point reading from a known concentration of cells.

Thyroxine ELISA

An ELISA was used to determine the amount of T₄ released into the supernatant of FRTL-5 cultures

from differing passage numbers. The quantitative measurements of T₄ released were performed using a competitive, horseradish peroxidase-based, rat-specific ELISA purchased from Endocrine Technologies, Inc. (Newark, CA, USA). Briefly, established follicular cultures (<80% confluent) in six-well plates that were at least 24 h post medium replacement were given 1 nM NaI prior to harvesting the supernatant; in the Cx32 down-regulation experiments 3 mM heptanol was added 1 h prior to adding NaI. Several incubation periods were tested and the maximum T₄ production was detected 6 h after the addition of NaI. Freezing also reduced the detectable levels of T₄. Therefore, the supernatants were centrifuged to remove any non-adherent cells and/or debris and tested immediately (e.g. without prior freezing). Replicates of 100 µl aliquots were mixed with a constant amount of peroxidase-conjugated T₄, and incubated in microtiter wells coated with anti-rat T₄ for 2 h at 22 °C, followed by washing with PBS containing 0.05% Tween-20 (Fisher Scientific, Tustin, CA, USA). After washing, 3,3',5,5'-tetramethyl benzidine (TMB) substrate was added to all wells, color development was complete within 20 min and the plates were read at 450 nm on an automated plate reader (Model Fluorite 1000, Dynex Technologies, Inc., Chantilly, VA, USA) equipped with Revelation software version 3.0. The intensity of the color formed was proportional to the amount of enzyme present and was inversely related to the amount of unlabeled T₄ in the sample. Complete cell medium alone was used in the blank wells. A series of T₄ standards assayed in the same way was used to construct a standard curve from which the concentrations of T₄ in the test supernatants were interpolated.

Later passage cultures produced fewer follicles per equivalent starting cell densities, so we normalized T₄ production per number of follicles in the cultures. Follicle counts were performed as previously described in Green *et al.* (2001). Briefly, plates/wells from which the supernatants were harvested were fixed with methanol:acetic acid (3:1 v/v) for 15 min at room temperature followed by crystal violet staining. Counting was performed using an Alpha Innotech image processing system with trans-illumination. The machine was calibrated and manually adjusted for the size threshold, which was confirmed by visual inspection. The size threshold was set to exclude any follicle or cell aggregate less than 100 µm in diameter. The automated counts were checked for precision by random sampling and comparison with manual counts. The precision limit was consistently within 5–7%.

Statistical analysis

Comparisons between early and late passage cells or samples were determined by Student's *t*-test. Proportional differences in the dye transfer experiments were judged for significance by Chi-squared analysis. $P < 0.5$ was considered the limit for statistical significance.

RESULTS

Gap junction-mediated cell-to-cell communication

When monolayer FRTL-5 cells were microinjected with Lucifer Yellow dye, a fluorescent tracer used to judge cell-to-cell communication by measuring the degree of dye transfer between contiguous neighboring cells, we found that they were uncoupled. The contacting, monolayer FRTL-5 cells transferred dye from the injected cell to a primary contacting cell in less than 2% of trial injections (Table 1). This lack of cell-to-cell communication, as judged by failure to transfer dye, did not change with age or passage number as long as the cells remained in a monolayer configuration (Fig. 1a and b).

When FRTL-5 cells established as follicles were microinjected, they transferred dye from the injected cell to second- and third-order neighboring cells and beyond within 2 min of injection (Table 1, Fig. 1c and d). This intercellular communication was manifest in follicular cultures that had been passed fewer than 15 times. Once the cultures had been maintained past passage 15 they only occasionally transferred dye to adjacent (first order) neighboring cells (Fig. 1e and f). Chi-squared analysis was performed comparing the proportional differences between each of the morphological states and dye transfer categories. The ability of early versus late follicles to transfer microinjected dye was significantly different ($P < 0.001$), as were early follicles to monolayers ($P < 0.001$).

Immunophenotyping of connexin expression

The presence of plasma membrane assembled gap junctions is a prerequisite to electrically coupled intercellular communication (Unwin & Zampighi 1980). FRTL-5 cells from early (<12) and late (>15) passages established as monolayers were immuno-labeled with anti-Cx43, Cx32 and Cx26 to determine whether proteins corresponding to the rat thyroid Cxs were expressed. The immuno-labeling revealed that none of the three connexins were expressed in monolayer FRTL-5 cells (data not

shown). Cx32 was detectable as a plasma membrane punctate structure in follicular cultures, but only in those passed fewer than 15 times (Fig. 2). The follicular organization did not allow for a definitive intracellular location of Cx32 to be visualized, so cryostat sections of follicles were immuno-labeled for the three connexins. A representative composite is shown in Fig. 3. Again Cx43 and Cx26 were not evident; however, Cx32 protein containing gap junctions were present in the lateral membranes of these passage 12 FRTL-5 cells. Later passage FRTL-5 follicle sections did not label with any of the anti-connexin antibodies/antisera (data not shown).

Western blot analyses for Cx32 protein

Connexin proteins can be expressed without being trafficked and/or assembled correctly in the plasma membrane at points of cell-to-cell contact (Green *et al.* 1995a, 1996, 1997, Deschenes *et al.* 1997). Therefore we fractionated the cell lysates and extracted protein from various passage number FRTL-5 cultures in an attempt to understand the loss of Cx32 protein expression in the later passages. Total, membrane and cytosolic components were separated, immunoprecipitated and electrophoresed in parallel. A representative Western blot is shown in Fig. 4. Cx32 protein expression was detected in early passage cultures (pass 5 and 12). Although the membrane-enriched fraction from pass 12 cultures was reduced in the amount of Cx32 detected, no Cx32 was detected in later passage cultures (pass 18 and 25). Thus the later passage cells were not expressing immuno-detectable Cx32. Total lysates of liver and lung tissue were run in parallel for positive and negative controls respectively.

Northern blot and RT-PCR analyses for connexin mRNA

To corroborate the functional (dye transfer) and immunocytochemical data we investigated transcriptional expression of Cx32 using isolated total RNA from passage 12 FRTL-5 cultures. The RNA was electrophoresed and the Northern blots hybridized with connexin specific cDNAs. The blots revealed that Cx32 RNA was present, however no Cx43 or Cx26 mRNAs were detected in the pass 12 FRTL-5 cells (data not shown).

The connexins are a family of related proteins that are highly conserved, single copy genes (Willecke *et al.* 1990). Cx32 is located on the X chromosome, and the gene has two exons, one in the 5' untranslated region and the other, exon 2,

FIGURE 4. Western blot analyses for Cx32 protein expression in FRTL-5 cells. Different passes of FRTL-5 cells were lysed and fractionated by ultra-centrifugation. Total protein lysate (lanes 2, 6, 9, 12) cytosolic (lanes 3, 7, 10, 13) and membrane (lanes 4, 8, 11, 14) enriched fractions were immunoprecipitated with anti-Cx32 antibody, followed by Western blot analyses. Cx32 protein expression was detected in early cultures (passes 5 and 12), whereas no Cx32 was detected in later passage cultures (passes 18 and 25). Molecular weight markers are in lane 1. Total protein from liver (lane 5) and lung (lane 15) tissue were included as positive and negative controls respectively.

contains the entire, uninterrupted coding sequence (Miller *et al.* 1988). The Cx32 protein is 283 amino acids in length, and has a cytoplasmic amino-terminus, four transmembrane regions, two extracellular loops, a cytoplasmic loop and a cytoplasmic carboxyl-tail region (Dahl *et al.* 1987). The most variability between family members occurs in the cytoplasmic loop and carboxyl-tail regions. To better understand the passage-dependent loss of Cx32 expression we designed primers for these unique regions of the rat Cx32 molecule for use in an RT-PCR assay. The RT-PCR DNA products corresponding to the cytoplasmic loop and the carboxyl-terminal region are shown in Fig. 5. Primary rat thyroid and early pass FRTL-5 cultured cells (pass 5 and 14) yielded bands that corresponded to the 81 bases of the cytoplasmic loop and 190 bases of the carboxyl-terminal region of rat Cx32 DNA. FRTL-5 cells from late passes produced a band only at the cytoplasmic loop position with no product at the C-terminal fragment position (Fig. 5).

To determine whether the electrophoresed bands were equivalent to the known rat Cx32 sequences, the FRTL-5 products were aligned using a BLAST search from the www.ncbi.nih.gov web site (ref/NM 017251.1/*Rattus norvegicus* gap junction membrane channel protein beta-1 (Gjb1), mRNA length=1485 nucleotides, coding region 32–881). The results are shown in Fig. 6. The primary thyroid and FRTL-5 cells from early passes produced cytoplasmic loop sequences that exactly matched the known rat sequence (base positions 343–424). The carboxyl-terminal sequence bases 23–213 of the RT-PCR product almost exactly matched the known rat sequence, nucleotide

positions 705–895. The only exception was a transversion of a G to a C at our position 164, which corresponded to known rat Cx32 sequence position base 850, amino acid number 273. This change had no net effect as both codons, CUC and CUG, specify leucine (Fig. 6B).

The later passage FRTL-5 cells had a single base deletion at position 36 of the 81 base-long DNA products of the cytoplasmic loop region (corresponding to base position 378 of the published sequence, Fig. 6A). The single base deletion altered the protein sequence starting at amino acid 116, where the normally occurring histidine was changed to a proline (Fig. 6A). The next four amino acids were changed from leucine, glutamic acid, glutamic acid and valine, to tryptophan, lysine, arginine and stop (UAA, ochre). The Cx32 protein stops at amino acid 120, which occurs at the site where the cytoplasmic loop starts its ascent toward the third transmembrane region. This premature truncation resulted in a protein that is less than half the length of the normal Cx32 protein. This finding is consistent with the immunocytochemical data. The lack of a RT-PCR product corresponding to the carboxyl-terminus in the later passage cultures indicates that the mRNA for Cx32 is also truncated. This truncation explains the failure to detect the mRNA for Cx32 by Northern blot analysis due to the significantly reduced strength of the hybridization signal.

FRTL-5 cell karyotype

The ubiquitous expression of connexin proteins, especially Cx43 in continuous passage cell lines suggested that there may be genetic or epigenetic

FIGURE 5. RT-PCR analyses of Cx32 mRNA. Specific primers were made to the Cx32 cytoplasmic loop region (resulting products in lanes 1–4), and the C-terminal region (products in lanes 5–8). DNA products from primary rat thyroid RNA are in lanes 1 and 5, FRTL-5 pass 5 in lanes 2 and 6, FRTL-5 pass 14 in lanes 3 and 7, and FRTL-5 pass 25 in lanes 4 and 8. Bands corresponding to the cytoplasmic loop position (approximately 100 bp) were detected in all samples loaded, whereas bands corresponding to the C-terminal position (approximately 200 bp) were present in all except pass 25, the latest FRTL-5 passage tested.

abnormalities that could account for the lack of Cx43 and Cx26 expression in FRTL-5 cells. Several reports describe FRTL-5 cells as being diploid (Mulcahy *et al.* 1985, Bellur *et al.* 1990, Dai *et al.* 1992, Backeljauw *et al.* 1993). When we inquired in late 1996 whether the ATCC had a record of the FRTL-5 karyotype they did not. Chromosome spreads from pass 3–12 of the FRTL-5 cell cultures revealed a complete tetraploid karyotype; a typical spread is shown in Fig. 7A. These spreads were consistently composed of 88 chromosomes which was four times the 22 haploid chromosomes normally found in rat cells ($n=50$ spreads). When late passage cultures (passes 18–22) were evaluated, they were found to be tetraploid plus 3 ± 1 extra chromosomes ($n=50$ spreads). The extra chromosomes found in these cultured cells were not always the same ones based on size and centromere position. Collectively there were no visible translocations, breaks or obvious losses of chromosomes or chromatids in any of the FRTL-5 cultures tested (Fig. 7B).

Morphologic changes as a function of FRTL-5 passage number

Under early passage conditions, FRTL-5 cells exist primarily as discrete follicles. However, as they are repeatedly passed they began to change. Cultures of FRTL-5 cells that are overgrown, or at a medium density but from a late pass number showed a monolayer 'skirting' that spreads from beneath the follicles. The follicles also become flatter, as if their luminal spaces were reduced in volume (Fig. 8). The shape of cells making up monolayer areas are flatter, larger and more cuboidal than cells organized, or participating, in follicles.

Growth rate as a function of FRTL-5 passage number

The reported average cell division time for FRTL-5 cells varies greatly in the literature with the most common doubling time reported as 30–36 h (Broising *et al.* 1989, Huber *et al.* 1990, Pekary *et al.* 1995, 1997, Statuto *et al.* 1997). From experience with FRTL-5 cells it was clear that older (higher passage number) cultures established and grew faster than early passes. To measure this, FRTL-5 cells of various passage numbers were compared (Fig. 9). We found that FRTL-5 cells from passes 5–8 had an average division time of 49 ± 1 h, and later passes 20–25 had an average doubling time of 28 ± 3 h. Intermediate passage numbered cultures had intermediate growth rates (Table 2).

Thyroxine release as a function of FRTL-5 passage number

Thyroxine levels were measured in early and late FRTL-5 cultures to determine whether the presence or absence of competent Cx32 gap junctions correlated with altered output of T_4 . As an additional way to assess the contribution of Cx32 gap junctions to T_4 release, we treated early passage follicular cultures with heptanol which binds to

FIGURE 6. RT-PCR product sequencing and alignment. Shown are the sequences from the 81 bp band of FRTL-5 pass 25 (panel A) and the 190 bp band of FRTL-5 pass 5 (panel B) that were purified and sequenced. The RT-PCR products were BLAST aligned with the known rat Cx32 (beta 1 protein) nucleotide sequence. The results revealed that the product from pass 25 cells corresponded to 80 of the 81 nucleotides of the known sequence positions 343–424 in the cytoplasmic loop region of rat Cx32. The missing base was a deletion at position 378 in the published sequence, our product number 36. The deleted 'a' caused a frame shift which altered the amino acid sequence at position 116 where the normally produced histidine (CAC) became a proline (CCC). All the FRTL-5 cultures produced RT-PCR products that had a transversion from a G to a C in the C-terminus (panel B). This transversion did not change the amino acid sequence, both CUG and CUC code for leucine. The dotted line (...) in panel B indicates bases that are not shown to conserve space; the dashed line (---) indicates that the query sequence identically matched the subject sequence; square brackets, [], indicate codons of interest; and parentheses, (), indicate translation stop codons. The numbers at the start and stop of the lines indicate the DNA, RNA or amino acid numbers of the sequences investigated.

A

ref/ NM 017251.1/ Rattus norvegicus gap junction membrane channel protein beta-1 (Gjb1), mRNA
length = 1485 strand = plus/plus Coding bases (32..881), protein_id= "CAA27705.1" (aa 1-283)

CYTOPLASMIC-LOOP

Sbj: DNA 343 a atgctacggcttgaggggcacggg gac ccc ctt [cac] ctg gaa gag gta aagaggcacaaaggtgcacatctcaggacactg424
Query: 9 ----- [c_cc]tgg aag agg (taa) agaggcacaaaggtgcacatctcaggacactg. 90

Sbj: RNA 343 a augcuacggcuugaggggcacggg gac ccc cuu [cac] cug gaa gag gua aagaggcacaaagugcacauucucagggacacug424
Query: 9 ----- [ccc] ugg aag agg (uaa) agaggcacaaagugcacauucagggacacug. 89

Sbj: aa105met-leu-arg-leu-glu-gly-his-gly-asp-pro-leu-[his]-leu-glu-glu-val-lys-arg-his-lys-val-his-ile-ser-gly129
Query: 105 -----[pro]-trp-lys-arg-(uaa) 120

Single base deletion in Query DNA at nucleotide # 36, which corresponds to base #379 of the subject sequence

B

>ref / NM 017251.1/ Rattus norvegicus gap junction membrane channel protein beta-1 (Gjb1), mRNA
length = 1485 strand = plus/plus Coding bases (32..881), protein_id= "CAA27705.1" (aa 1-283)

CARBOXYL-TERMINUS

Sbj: DNA 705ccaatccgcctcccgcaaggctcggg.....gagatcaacaagctgtgagcgagcaggatggctcttgaagacatactgcgcgcagt830
Query: 23 ----- 138

Sbj: DNA 831 cct ggc act ggg gcc ggg [ctg] gct gag aag agc gac cga tgc tca gcc tgc (tga) tgcgagtagcca 895
Query: 139 ----- [ctc] ----- (tga) ----- 213

Sbj: RNA 831 ccu ggc acu ggg gcc ggg [cug] gcu gag aag agc gac cga ugc uca gcc ugc (uga) ugccgaguacca 895
Query: 139 ----- [cuc] ----- (uga) ----- 213

Sbj: aa 267 -pro-gly-thr-gly-ala-gly-[leu] ala-glu-lys-ser-asp-arg-cys-ser-ala-cys-(uga)284
Query: 267 -----[leu] ----- (uga)284

Transversion of G to a C at Query # 168 which corresponds to base # 850 of the subject sequence

FIGURE 7. FRTL-5 cell karyotype. Chromosome spreads were prepared from various passage FRTL-5 cultures. Panel A shows a typical chromosome spread from early passage cultures (passes 3–15) of FRTL-5 cells. When arranged by size and centromere position they were found to be fully tetraploid with four times 22 chromosomes for a total of 88 (panel B). Later passage cultures (passes 15–22) were typically tetraploid plus two or three extra chromosomes (data not shown).

lipid cholesterol causing a conformation change in the connexins that results in the closing of the junctions (Bastiaanse *et al.* 1993). Closed junctions are removed from the plasma membrane and degraded (Jordan *et al.* 2001). New Cx32 protein is made and gap junctions are reassembled. The total time from addition of heptanol to re-established communication is approximately 6 h. Thyroxine levels were measured using a colorimetric rat-specific competitive ELISA. Because later passage cultures produced fewer follicles under conditions of equivalent starting cell densities, we normalized the level of T_4 released on a per follicle basis. Early

cultures treated for 6 h with NaI released to the supernatant an average of 5.27 ± 0.33 ng/ml T_4 per 100 follicles, early passage cultures treated with heptanol released an average of 3.91 ± 0.55 , whereas later passage cultures released 3.77 ± 0.45 ng/ml T_4 per 100 follicles. Treatment of later passage follicular cultures with heptanol did not significantly alter the level of T_4 released. The results for the T_4 data are compiled in Table 3. The difference in T_4 release between Cx32 competent and Cx32 incompetent follicular cultures was 27% and was statistically significant ($P < 0.0001$) and similar to the drop in T_4 levels in hypothyroid rats (35% decrease reported by Green *et al.* 1996). When tested at 24 h, the levels of T_4 were approximately 60% less than detected in the same flasks at 6 h. Freezing also reduced the detectable levels of T_4 .

Control conditions for these experiments included T_4 release in the absence of iodide, and from cell monolayers versus cells organized as follicles. Thyroxine was not detected in supernatants from monolayers of early or late FRTL-5 passages, with or without iodide. Early and late FRTL-5 follicles produced T_4 at very low levels when cultured in the absence of iodide. The early FRTL-5 follicles released 1.13 ± 0.21 ng/ml T_4 per 100 follicles and the late FRTL-5 follicles released 0.22 ± 0.04 ng/ml T_4 per 100 follicles into the culture supernatant (Table 3).

TABLE 2. Growth rate in FRTL-5 cells as a function of passage number

	No. of divisions/ 24 h	Doubling time (h)	n ^a
Pass 6 ^b	0.48	49 ± 1	3
Pass 9	0.52	46 ± 2	2
Pass 13	0.55	44 ± 1	3
Pass 17	0.67	36 ± 2	3
Pass 20	0.86	28 ± 3	3
Pass 25	0.96	25 ± 2	2

^aNumber of experiments (eight replicates per experiment).
^bNumber of times FRTL-5 cells were passaged in our laboratory.

FIGURE 8. Morphologic changes in FRTL-5 cells as a function of passage number. When maintained normally, early passage FRTL-5 cultures organized into discrete follicles usually surrounded by cell-free areas. Shown is a pass 6 culture photographed on day 5 (panel a) and on day 11 (panel b). When the cultures are left past the normal passage time (panel c is at 13 days), or are derived from later passages (shown is pass 19) they develop a monolayer 'skirting' that spreads from beneath the follicles (panel d). Note also the flattened appearance of the follicles in the later passage cultures, which suggests a reduced luminal volume (panel d). Capture magnification of panels a–c was $10\times$ and panel d $40\times$.

DISCUSSION

Our use of FRTL-5 cells has been as a model system in which we have developed protocols for use with primary thyroid cultures. As such we have kept careful passage records utilizing only early passages for the retention of normal thyrocyte characteristics. The only significant culture condition change we introduced was to use DMEM:F12 instead of F12 medium. Numerous reports by others ascribed properties to FRTL-5 cells that differed from what we observed. This prompted us to continue expanding the cultures to determine whether age-related changes or conditional differences could explain the discrepant results.

Follicular cultures of FRTL-5 cells are coupled by Cx32-type gap junctions. They transfer Lucifer

Yellow dye from an injected cell to third-order neighboring contacts and beyond, and have discrete immuno-detectable punctate fluorescent plaques corresponding to Cx32 gap junctions at their lateral contacting plasma membrane surfaces. Inter cellular communication via Cx32 expression was only manifest in cultures that were maintained as follicles and passed fewer than 15 times. Microinjection, immunocytochemistry, Western and Northern blot analysis confirmed that once cultures were passed more than 15 times they did not communicate, as determined by dye transfer, nor did they contain detectable Cx32 mRNA or protein.

To better understand this demarcation of Cx32 expression at passage 15 we used RT-PCR with primers generated to amplify the two most unique regions of the rat Cx32 DNA sequence (Paul 1986, Beyer *et al.* 1987, Zimmer *et al.* 1987, Miller *et al.*

FIGURE 9. Growth rate as a function of FRTL-5 passage number. FRTL-5 cells from different passes (6, 13, 17, 20) are shown. The cells were seeded at 2×10^4 cells per well in 96-well plates and titered by twofold serial dilution. At the indicated times, cell density was determined using bio-reduction of MTS. The graph represents the number of cell divisions versus time in hours for the various passage numbered cultures shown. Each *symbol* represents the mean of 24 ($=3 \times 8$) replicate determinations $\pm$ s.e. The resultant doubling times are shown here and the growth rate values listed in Table 2.

TABLE 3. Thyroxine release by FRTL-5 cells

	6 h ^a			24 h			
	T ₄ ^b	Follicle count ^c	T ₄ per/100 follicles ^d	T ₄	Follicle count	T ₄ per/100 follicles	n ^e
<i>Early^f</i>							
Follicle ^g +I ^h	39.65 $\pm$ 2.6	752	5.27 $\pm$ 0.3	15.86 $\pm$ 0.98	752	2.11 $\pm$ 0.13	4
Follicle+H ⁱ +I	24.35 $\pm$ 3.0	623	3.91 $\pm$ 0.6	NT	—	—	2
Follicle – I ^j	5.94 $\pm$ 1.1	528	1.13 $\pm$ 0.2	2.38 $\pm$ 0.49	528	0.45 $\pm$ 0.10	3
Follicle+H – I	2.98 $\pm$ 1.0	467	0.63 $\pm$ 0.2	NT	—	—	2
Monolayer ^k +I	00	—	00	00	—	00	2
Monolayer – I	00	—	00	00	—	00	2
<i>Late^l</i>							
Follicle+I	27.55 $\pm$ 3.3	731	3.77 $\pm$ 0.5	11.20 $\pm$ 1.3	731	1.53 $\pm$ 0.18	4
Follicle+H+I	21.86 $\pm$ 2.9	599	3.65 $\pm$ 0.5	NT	—	—	2
Follicle – I	1.08 $\pm$ 0.2	498	0.22 $\pm$ 0.0	00	498	00	3
Follicle+H – I	1.65 $\pm$ 0.6	551	0.29 $\pm$ 0.1	NT	—	—	2
Monolayer+I	00	—	00	00	—	00	2
Monolayer – I	00	—	00	00	—	00	2

^aTime cultures were incubated with 1 nM NaI.
^bThyroxine concentration ng/ml (all cultures in six-well plates were in 1 ml volumes).
^cTotal follicles from which T₄ was measured.
^dNormalizing of T₄ concentration per 100 follicles.
^eNumber of experiments, each with duplicate samples.
^fFRTL-5 cells passed from 5 to 12 times.
^gCultures grown as organized follicles.
^hSodium iodide, used 1 μ M stock solution, final concentration 1 nM.
ⁱ3 mM heptanol added 1 h prior to adding NaI.
^jNo NaI was added.
^kCultures dispersed and grown as a single cell layer.
^lFRTL-5 cells passed from 16–25 times.
NT, Not tested.

1988). RT-PCR products from FRTL-5 RNA revealed that cultures past passage 15 had acquired a single base deletion in the middle of the cytoplasmic loop region and did not make an RT-PCR product corresponding to the C-terminus. This point deletion caused a frame shift that resulted in a translation stop signal four codons or 12 nucleotides downstream from the deleted base. This premature termination of the protein at amino acid 120 resulted in a failure to complete the remaining 60% of the molecule. Even with the frame shift that resulted in truncation of the protein, the entire sequence for the cytoplasmic loop region was made into a DNA product by RT-PCR. However, the lack of a sequence corresponding to the carboxyl-tail region suggests that the mRNA for Cx32 is also truncated. The message truncation must occur after the cytoplasmic loop between the third transmembrane region and start of the carboxyl-terminus. The altered protein sequence and loss of amino acids provides an obvious reason for the lack of antibody recognition, and failure to immuno-detect Cx32 protein in the later passage cultured cells. The failure of Northern blot analysis to detect Cx32 mRNA in the later passes is in agreement with a truncation. The loss of a significant portion of the

mRNA would result in a reduction in the stringency required for hybridization and under normal conditions the smaller and reduced signal would be lost. This explains our failure to detect Cx32 mRNA in later passage cultures under conditions wherein primary thyroid and control liver gave strong hybridization signals.

There is a precedent for mutations in the Cx32 gene contributing to the pathogenesis of inherited demyelinating neuropathy in X-linked Charcot-Marie-Tooth disease (Balice-Gordon *et al.* 1998, Scherer *et al.* 1999). Cx32 localizes to the paranodes and incisures of myelinating Schwann cells, where it is thought to participate in diffusion of ions and small molecules directly across the myelin sheaths (Scherer *et al.* 1999). The lack of functional Cx32 in this disease is associated with decreased nerve conduction velocities (Rouger *et al.* 1997), and non-functional Cx32 that is incorrectly trafficked accumulating in the cytosol causing toxicity (Deschenes *et al.* 1997, Scherer *et al.* 1999). There are numerous documented mutations in the coding region of the Cx32 gene, the most applicable examples include nonsense mutations in three different families that had transition mutations at amino acid 22 (arginine to stop) and transversion mutations at amino acid 186 (glutamate to stop) (Ionasescu *et al.* 1994), additional sites of point mutations are amino acid 15, 60, 63, 142, 208 and 215 (Fairweather *et al.* 1994, Ionasescu *et al.* 1994). Frame shift, point deletions and mutations have been identified in every domain of the Cx32 molecule and lead to incorrect trafficking, reduction and abrogation of Cx32 function in different families with this X-linked, inherited disease. These mutations provide evidence that each of the protein domains is important to the correct processing and function of Cx32 (Deschenes *et al.* 1997).

The finding of truncated Cx32 with age suggests that the observation by Statuto *et al.* (1997), that FRTL-5 cells do not express Cx32 mRNA or protein, could be due to the age or passage number of their cultures. We suggest that their cultures were past the equivalent of our passage 15 at the time of the study, perhaps because their original isolate may have been older. This supposition is consistent with the growth rate they report for their FRTL-5 cultures. It should also be noted that the hormone supplement somatostatin was eliminated from their culture medium. According to Ambesi-Impiombato *et al.* (1980), the presence of somatostatin was most important to cells plated at very low densities, and when absent the cells grew more slowly. They implied that under normal culture conditions the role for somatostatin was less critical, although recent studies suggest that

FRTL-5 cells can make somatostatin (Medina *et al.* 1999) and that somatostatin and TSH have opposing effects that act to balance the cell cycle regulating components leading to proliferation (Azuma *et al.* 1990, Medina *et al.* 2000).

Cx32 expression is transient in primary cells (Neveu *et al.* 1994) and rarely found in cell lines whether continuous passage or transformed. In fact, hepatocytes, which should express Cx32 as their dominant gap junction protein, when established in culture, quickly lose Cx32 and become Cx43 gap junction coupled cells (Neveu *et al.* 1995). Interestingly, Kojima *et al.* (1995) demonstrated that changing the medium and serum conditions induced the reappearance and long-term maintenance of Cx32 in adult rat hepatocytes. The retention of Cx32 by early passage FRTL-5 cells provides a unique opportunity for investigating the role of Cx32 and its contribution to normal thyrocyte properties.

When we purchased FRTL-5 cells we were focused on gap junctions and contact-dependent disturbances associated with inflammation in primary thyroid cultures. We assumed that their ability to transfer Lucifer Yellow dye to contacting neighboring cells was due to the ubiquitous expression of Cx43 type gap junctions. When FRTL-5 cells did not express Cx43 we conducted our first karyotypic examination of these cells to determine whether they had any genotype changes that could account for this loss. Our cultures in 1995 were fully tetraploid (modal number 88) as were cells from an original vial received from the ATCC (1992). Thus, the absence of Cx43 could not be explained by a large-scale loss of chromosomal DNA. However, any number of small gene mutations and/or altered regulatory mechanisms might explain its down-regulation as we found for Cx32.

In the original report by Ambesi-Impiombato *et al.* (1980) the FRTL-5 cells were checked and reported as having a diploid chromosome number. Since then, there have been several reports that FRTL-5 cells are diploid (Mulcahy *et al.* 1985, Bellur *et al.* 1990, Dai *et al.* 1992, Backeljauw *et al.* 1993). Most of these studies do not appear to have included a direct measurement of chromosome number, but rather reported a characteristic that had been associated with this fairly normal thyroid cell line. In 1998, Tasevski *et al.* reported that pass 14 FRTL-5 cells obtained from the ATCC were non-diploid with a modal number of 84 chromosomes (Tasevski *et al.* 1998). In that report fetal calf serum was used to grow the FRTL-5 cells. This would produce a set of cellular responses driven by the rich growth factor environment of fetal serum

and preclude a direct comparison with cells grown in a more defined media. Under our culture conditions the tetraploid genotype of FRTL-5 cells is consistent with normal cellular responses and state of thyroid differentiation, and suggests a balance of gene regulation and expression. Tetraploidy *per se* can be consistent with normal cell behavior and is supported by a report of a tetraploid species of rat (Gallardo *et al.* 1999). When later passage cultures of FRTL-5 cells were examined they were found to be aneuploid (tetraploid plus two or three extra chromosomes) and to have aberrant properties. The reason for this genomic instability is currently unknown.

An additional change that became evident with repeated passage was an increase in the degree of monolayer growth and subsequent reduction in the follicular content of the cultures. These tissue organization changes were coincident with the loss of functional Cx32, which supports a potential relationship between the loss of communication and the change in morphogenic appearance, as has been previously suggested (Munari-Silem *et al.* 1994). Of course, accompanying changes in adhesion properties and cytoskeletal components are also possible. This loss of functional tissue unit organization with age could explain reports that FRTL-5 cells do not form follicles (Zurzolo *et al.* 1991, Statuto *et al.* 1997, Tonoli *et al.* 2000) and implies that the cultures being used are well past passage 15. Associated with these morphologic changes was a dramatic increase in growth rate of the older (higher pass number) cultures. Our early pass cultures divided on average every 49 h, a value similar to primary thyroid cells (Christov *et al.* 1973, Green *et al.* 1996). Most often, the average division time reported ranged from 30 to 36 h (Brosing *et al.* 1989, Huber *et al.* 1990, Pekary *et al.* 1995, 1997, Statuto *et al.* 1997). From our study this would correspond to cultures that had been advanced beyond pass 17. Although passage number is strongly implicated, we cannot rule out the possibility that the medium and other conditional differences between laboratories could be responsible for some of the different properties evident at earlier passage numbers. An example of how the growth rate of FRTL-5 cells can be used as an indicator of passage number, and demonstrate the relationship of Cx32 expression to growth control is exemplified in the report by Statuto *et al.* (1997). They measured the wild-type division time for their FRTL-5 cells at 35 h and found that FRTL-5 cells did not express Cx32 at the mRNA or protein levels. According to our growth curves their cells would be at about passage 17 and would not be expressing full length Cx32. Interestingly, in their

study they up-regulate Cx32 expression by transfection and found the division time was now extended to 50 h. This protracted time per cell division with the re-introduction of competent Cx32 gap junctions parallels the properties of the Cx32 expressing FRTL-5 follicular cultures we defined as early (passes 3–12) and argues strongly for the casual link between Cx32 expression and the other phenotypic changes. This dramatic change in growth rate with the expression of Cx32 supports its potential role as a tumor suppressor (Kerbel 1995, Mehta *et al.* 1999, Yamasaki *et al.* 1999a,b) and underscores its importance in maintaining organized, functional thyroid cells.

To complete this investigation we assessed a normal property of functional thyroid follicles by measuring T_4 release. The 27% difference in T_4 release between early and later passage follicles is consistent with the idea that homotypic coupling between glandular epithelium increases the output of hormone, as was described in pancreatic islet cells by Meda (1989). In our case, coordinated follicular thyrocytes that are coupled by gap junctions have an increased output of thyroid hormone on a per follicle basis when compared with follicles that are no longer coupled by Cx32-type gap junctions. Support for this contention was obtained when heptanol treatment of early cultures (to close the channels) resulted in levels of T_4 similar to late passage cultures that have a mutated, non-functional form of Cx32. Although other factors may be contributing to this reduction, a similar situation exists in primary thyrocytes isolated from sites of autoimmune thyroiditis wherein the thyroid specific gap junctions are produced but not trafficked correctly and so remain cytosolic (Green *et al.* 1995a, 1996, 1997). In these uncoupled primary thyroid follicles, the hypothyroidism is, in part, due to the lack of coordinated responses to TSH stimulation. In light of the mutations in Cx32 found in this study and those described for the X-linked Charcot–Marie–Tooth disease (Deschenes *et al.* 1997), the abnormal assembly of gap junctions in autoimmune thyroiditis should be revisited.

Collectively, FRTL-5 cells, especially from early passes, retain a differentiated thyroid phenotype with respect to both their physical and functional properties. Clearly, there is a progressive loss of thyroid differentiation with repeated passage, we however did not achieve a completely dedifferentiated phenotype by passage 25, the latest passage tested in this study. We believe that there will be a limit to the dedifferentiation and that eventually the FRTL-5 cells will reach a stable point and persist. The differences between early and late passage numbered cultures, characterized in this study,

explain many of the unqualified features that have been ascribed to FRTL-5 cells. We found, under the conditions specified, that FRTL-5 cells from passes 3–15 form functional thyroid follicles, and display normal thyroid properties, including expression of Cx32 gap junctions, have a growth rate similar to primary thyrocytes and release T₄. Therefore, early passage cultures provide an excellent model to study thyroid function *in vitro*.

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