Cellular distribution of *Egr1* transcription in the male rat pituitary gland.

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Abstract

The transcription factor gene, *Egr1,* is necessary for female fertility; EGR1 protein is an established molecular regulator of adult female gonadotroph function where it mediates GnRH-stimulated transcription of the *Lhb* gene. Recent studies have also implicated pituitary EGR1 in the mediation of other physiological signals suggesting an integrative function. However, the role of EGR1 in males is less well defined and this uncertainty is compounded by the absence of cellular expression data in the male pituitary gland. The aim of the present study, therefore, was to define the distribution of *Egr1* gene expression in the adult male rat pituitary. To further this aim, we have evaluated cellular populations in a transgenic rat model (*Egr1-d2EGFP*) in which we demonstrate regulated GFP (green fluorescent protein) expression in EGR1+ pituitary cells. Cellular filling by GFP enabled morphological and molecular differentiation of different populations of gonadotrophs; *Egr1* transcription and LHB were highly co-localized in a major population of large cells but only minimally co-localized in small GFP+ cells; the latter cells were shown to be largely (80%) composed of minority populations of GH+ somatotrophs (9% of total GH+) and PRL+ lactotrophs (3% of total PRL+). *Egr1* transcription was not found in TSH+, ACTH+ or SOX2+ precursor cells and was only minimally co-localized in S-100β+ folliculostellate cells. Our demonstration that the *Egr1* gene is actively and selectively transcribed in a major sub-population of male LHB+ cells indicates a largely conserved role in gonadotroph function and has provided a basis for further defining this role.

Introduction

Cell-specific molecular mechanisms regulate anterior pituitary hormone production; each hormone cell-type also has distinct sets of developmental and physiological regulatory factors (as reviewed by Perez-Castro et al, 2012). One important molecular regulator in the adult pituitary is the C2H2 zinc finger transcription factor, EGR1 (early growth response factor 1), that is expressed as a 75kDa nuclear protein in the rat anterior pituitary (Tremblay & Drouin, 1999; Wolfe & Call, 1999; Slade & Carter, 2000; Knight et al, 2000). A requirement for EGR1 in the control of gonadotroph function was revealed by gene knockout in mice (Lee et al, 1996; Topilko et al, 1998) and supported by a substantial body of data showing that EGR1 is a transcriptional regulator of the *Lhb* gene (luteinizing hormone beta-subunit gene; Tremblay & Drouin, 1999; Wolfe & Call, 1999; Slade & Carter, 2000), acting in association with other nuclear factors (as reviewed by Thackray et al, 2010; Miller et al, 2012).

EGR1 acts as a mediator of GnRH-induced *Lhb* transcription (Tremblay & Drouin, 1999; Wolfe & Call, 1999), and also appears to integrate other physiological inputs at a pituitary level. These include insulin and fatty acids (Buggs et al, 2006; Garrel et al, 2014), indicating a potential role in mediating effects of nutritional status and possibly obesity on reproductive capacity (see Brothers et al, 2010). Additionally, there is evidence that EGR1 mediates effects of glucocorticoids/stress at the *Lhb* gene promoter (Breen et al, 2012). EGR1 also appears to mediate intra-pituitary actions of kisspeptin on *Lhb* expression (Witham et al, 2013). Overall, therefore, EGR1 has been identified as a potential mediator of multiple different endocrine signals at the level of the gonadotroph.

The relatively restricted endocrine phenotype of *Egr1* null mutants (Lee et al, 1996; Topilko et al, 1998) is indicative of cellular specificity of EGR1 expression. We have

confirmed that EGR1 protein is highly restricted to LHB-expressing cells in the female rat anterior pituitary (Knight et al, 2000). In addition, we have generated *Egr1* transgenic rat models and have demonstrated that rat *Egr1* genomic (promoter and intron I) sequence directs transgene sequence to *Lhb* pituitary cells (Man & Carter, 2003), indicating that the pattern of pituitary expression is largely transcriptionally determined. Our understanding of anterior pituitary *Egr1* expression is incomplete, however, because previous cellular localization studies (Knight et al, 2000; Man & Carter, 2003) have been conducted in proestrous female rats and therefore potential differences in expression both across the oestrous cycle and in male rats are undefined. This is particularly relevant in the case of males because data from one *Egr1* null mutant model (Lee et al, 1996) indicates a sex difference in phenotype in that only females are infertile, arguing for a sex-specific role for *Egr1*. However, in an independently derived null-mutant model, males are also affected (Topilko et al, 1998). This difference has been attributed to mouse strain background (see Tourtellotte et al, 2000), coupled with an involvement of (male-specific) redundancy of *Egr1* with *Egr4* (Tourtellotte et al, 2000). It is also clear that sex differences in functional necessity for *Egr1* could reflect sex differences in the dynamics of this transcription factor. Thus, in females, there appears to be a requirement for phasic (and gene-specific) up-regulation of EGR1 in gonadotrophs during the pre-ovulatory stage of the oestrous cycle (Lee et al, 1996; Topilko et al, 1998; Slade & Carter, 2000). In males, however, there are likely to be no phasic actions of EGR1 in gonadotrophs, rather EGR1 may have only basal activity in these cells. Currently, the absence of data on Egr1 in the male pituitary is confounding investigation of these alternatives and consequently we have now conducted a detailed analysis of Egr1 transcription in the male rat pituitary.

In the present study we used a second generation *Egr1* transgenic rat model generated

in our laboratory (*Egr1-d2EGFP;* Man et al, 2007). This model retains a rat *Egr1* promoter sequence used in our first generation model (Man & Carter, 2003) but does not include *Egr1* intronic sequence. We have shown that the *Egr1-d2EGFP* model exhibits cell-specific expression of a destabilized green fluorescent protein variant (d2EGFP) in a range of tissues including the pituitary (Man et al, 2007; present study). A feature of this model is cellular-filling by the non-native GFP molecule that both enhances visualization of transgene expression and, importantly, provides an aid to cellular classification and identification by revealing cellular morphology that is absent in (nuclear) EGR1 detection. Using this model, we have therefore aimed to characterize GFP/EGR1 expression in the male rat anterior pituitary, determining the pattern of co-localization in both hormone-producing, and also other pituitary cell types. In this way, we hope to reveal if apparent sex differences in *Egr1* function are related to male-specific expression in the anterior pituitary gland.

Methods

Animals and tissue sampling

Animal studies were conducted in accordance with the Animal (Scientific Procedures) Act 1986, and local (Cardiff University) ethical review. Rats were maintained in a 14:10 light:dark cycle (lights on: 05.00h) in conventional rat cages (2-5/cage) with standard rat chow and water freely available. Health status was monitored frequently and assessed by veterinarian consultation if required. Transgenic rats of the *Egr1-d2EGFP* line (Man et al, 2007) were maintained on a Sprague-Dawley background by breeding hemizygote transgenic males with wild-type females (Charles River UK, Margate, Kent). For the majority of studies, the genotype of offspring was determined by PCR analysis of tissue biopsies (Man et al, 2007), and hemizygote females and males were selected for the current experiments. In one experiment only, offspring from a transgenic/wild-type cross

litter were killed on postnatal day 5 (P5, prior to genotyping) for direct analysis of transgene expression. In this case, a *post-mortem* tissue sample was taken for genotype analysis and confirmation of transgenic status. For direct analysis of transgene fluorescence, whole dissected pituitary glands (P5 and P20) were rinsed in phosphate buffered saline (PBS), positioned on microscope slides and imaged using a 2.5X objective and 'GFP' optics (excitation filter: BP 470/40; dichromatic mirror: 500; suppression filter: BP 525/50) on an epifluorescence microscope (Leica DM-LB, Leica Microsystems Imaging Solutions Ltd, Cambridge, UK).

Female rats for immunoblot analysis of pituitary transgene expression were selected for analysis on certain days of the oestrous cycle, determined by recording at least two full cycles as assessed by microscopic examination of cell populations in vaginal washings. Females, and males of equivalent age (5 months) were killed at 12.00h, and pituitary glands were dissected for protein extraction and analysis (Holter et al, 2008). Male rats for immunohistochemical analysis (adult, 5-6 months old) were terminally anaesthetized with sodium pentobarbitone (150 mg/kg, i.p., 17.00h) and perfused via the ascending aorta with phosphate buffered saline, followed by 4% paraformaldehyde in 0.1M phosphate buffer (PFA). Dissected whole pituitary glands were post-fixed in PFA overnight at 4˚C, and then cryoprotected in 20% sucrose in 0.1M phosphate buffer at 4˚C overnight. The glands were suspended in tin-foil wells of CRYO-M-BED (Bright Instrument Company Ltd, Huntingdon, UK) and frozen at -80˚C prior to sectioning.

Immunoblot analysis

Western immunoblot analysis was conducted as described previously (Holter et al, 2008), using whole cell extracts (60µg protein/lane). Whole cell extracts were obtained by homogenizing pituitary glands to a paste in ice-cold buffer (20 mM Hepes, pH 7.9, 1.5

mM $MgCl_2$, 0.42 M NaCl, 0.2 mM EDTA, 0.5 mM dithiothreitol and 25% glycerol together with protease (P8340, Sigma, Bournemouth, UK) and phosphatase (P2850, Sigma) inhibitor cocktails at the manufacturer's suggested dilution) using a glass pestle in a 1.5ml microtube. Homogenates were centrifuged (12,000rpm, 10 s), vortexed briefly, frozen on dry ice for 5 mins and then incubated on wet ice for 15 mins. Finally, homogenates were centrifuged again (12,000rpm, 15 mins, 4˚C) and supernatants were removed and stored at -80 ˚C. The total protein content of the extracts was estimated using the QuickStart™ Bradford reagent (BioRad, Hercules, CA, USA). d2EGFP protein was detected with a monoclonal antibody (632375; Clontech Laboratories Inc., Mountain View, CA, USA). Purified recombinant GFP protein (BD Biosciences, San Jose, CA, USA) was used as a positive control, and protein samples from non-transgenic animals served as a negative control. Western blots were re-probed with anti-ACTB (ab8227; Abcam, Cambridge, UK). Quantitative estimations of d2EGFP protein levels relative to levels of ACTB were obtained by densitometry (ImageQuant™ software 3.0, GE Healthcare, Little Chalfont, UK).

Immunohistochemical analysis

Sections of rat pituitary gland were cut in the transverse plane (12µm; Bright OTF cryostat with Magnacut knife (Bright) and mounted on glass slides (SuperFrost Plus, VWR International, Poole, Dorset, UK). Slides were dried briefly, and stored at -80 ºC prior to immunohistochemistry. GFP and various endogenous proteins were detected by fluorescence immunohistochemistry using procedures established in our laboratory (Holter et al, 2008). The following primary antisera, diluted in PBS-T (0.15% Triton X-100 in PBS), were used for non-hormone proteins: (chicken) anti-GFP, ab13970, Abcam, 1/400; (rabbit) anti-EGR1, 15F7, Cell Signalling Technology, Beverly, MA, USA, 1/400; (rabbit) anti-SOX2, 39823, Active Motif, Carlsbad, CA, USA, 1/1000; (mouse) anti-S-

100β□□SH-B1, Sigma, St Louis, MO, USA, 1/1000. Specificity of the EGR1 and S-100β antisera has been verified in a previous publication (Wells et al, 2011). Specificity of the SOX2 antisera was verified by demonstrating similar SOX2 detection to a previously verified antibody (Wells et al, 2011). Efficacy and specificity of the chicken antibody for GFP detection in the current application was verified in preliminary studies (see below). Hormone proteins were detected using custom antisera produced for this purpose by the Pituitary Hormones and Antisera Center (see Acknowledgements): ACTH, AFP-156102789, 1/100; FSHB, AFP-77981289, 1/100; GH, AFP-5672099, 1/100; LHB, AFP-C697071P, 1/100; PRL, AFP-4251091, 1/200; TSHB, AFP-1274789, 1/100. These hormone antisera were generated using highly purified immunogens, giving minimal cross-reactivity (datasheets available at www.humc.edu/hormones/material) and have been validated by antigen absorption in previous immunocytochemical analyses of the rodent pituitary (Yin et al, 2008). The specificity of these antisera was verified in the current study by showing that each antibody detected a selective population of cells in the anterior pituitary gland, but did not detect antigens in the neurointermediate lobe of the pituitary (exemplar LH data shown in Fig.4).

The primary antisera listed above were used in combination with appropriate species-specific, fluorophore-tagged, secondary antisera: Cy3-conjugated sheep anti-rabbit IgG, Sigma; Cy3-conjugated donkey anti-mouse IgG, Jackson Immunoresearch Laboratories Inc., West Grove, PA, USA; Alexa Fluor 488-conjugated goat anti-chicken IgG, abcam. Following washing, sections were sealed under coverslips using Vectashield with DAPI (Vector Laboratories, Burlingame, CA, USA). Pituitary sections were viewed using an fluorescence microscope (Leica DM-LB). Images were captured using a Leica DFC-

300FX digital camera and Leica QWin software (V3), and montaged in Photoshop (CS2, Adobe Systems Inc., San Jose, CA, USA).

Sections were selected for cell counting at a 'mid'-transverse level with (approximately) maximal lateral width of both anterior and neural lobes. The design for cell counting was similar to our previous publication (Knight et al, 2000; n=6 cell groups taken from 2 sections cut from each of 3 pituitary glands. However, in the current study each 'cell group' was generally a randomly selected group of 50 (defined) cells rather than microscope fields; this is because individual cells were sometimes indistinguishable when detecting particular antigens such as GH. The exception to this protocol was the counting of total anterior pituitary GFP+ve cells where entire (x40) fields were counted. In this case, images of microscope fields for both GFP and DAPI were captured and cells were counted using Adobe Photoshop tools to label cells and avoid double counting.

Statistical Analysis

Statistical comparison of experimental groups was conducted using Student t test for independent samples ($p<0.05$ significance level; SPSS 20, SPSS Inc., Chicago, ILL, USA).

Results

In initial studies, we confirmed expression of the *Egr1-d2EGFP* transgene in rat pituitary gland using GFP immunoblot analysis (Holter et al, 2008). In two independent transgenic lines, this analysis demonstrated both restriction of GFP immunoreactivity (indicative of Egr1 transcription) to transgenic animals (vs. wild-type) and also sex- and oestrous cycle-dependent expression of the transgene (Fig.1A, results for one transgenic line are

shown). Across three sample groups (male, female proestrus [P], female diestrus day 1 [D1, metestrus]), pituitary GFP levels were found to be highest in proestrous females (Fig.1.) and lowest in males (Fig.1A.). Lower levels of *Egr1* transcription in males compared with D1 females accords with previous analysis of *Egr1* mRNA (Slade & Carter, 2000). Notably, this analysis also reveals remarkably uniform expression levels (notwithstanding apparent biological variation in ACTB levels) across individual female transgenic rats that is suggestive of a tight association between *Egr1* transcription and the physiological rhythm of the rat oestrous cycle.

Next, we confirmed localization of *Egr1* transgene expression to the pituitary gland using both direct detection of GFP fluorescence and immunohistochemical detection of GFP protein (Fig.2). Direct detection of GFP fluorescence in developing rat pituitary glands revealed apparent pan-pituitary expression on postnatal day 5 (P5, Fig. 2A), a pattern of expression that retracted to anterior/intermediate pituitary expression on P20 (Fig. 2B). This developmental progression (of EGR1) has been observed previously in the mouse pituitary gland (Topilko et al, 1998). Although direct detection of GFP fluorescence is clearly feasible in this transgenic model, the requirement for extensive analysis at the cellular level demands that immunohistochemical procedures are used for quantitative co-localization studies. Immunohistochemical analysis of the rat anterior pituitary gland is greatly facilitated by the provision of custom antisera that are specific for the various hormone sub-units (see Acknowledgements), however, the use of these rabbit antisera necessitates the use of alternative species antisera for the detection of both EGR1 (Wells et al, 2011) and GFP (Holter et al, 2008) in co-localization studies. Accordingly, we characterized a chicken GFP antibody for this purpose, showing that this antibody detected a sub-population of rat anterior pituitary cells (Fig.2C) that matched expression of EGR1 protein with respect to both cellular localization and expression level (Fig.2D-F).

In non-transgenic animals, the chicken GFP antibody did not detect protein above background levels (data not shown). The sub-population of pituitary cells detected by GFP/EGR1 antisera in transgenics was approximately 5% of total DAPI+ cells (5.4% ± 0.35, n=6 x40 fields). As observed for pituitary EGR1 distribution (Knight et al, 2000), the GFP+ve cells were not uniformly scattered across the anterior pituitary but often seen collected into small groups of cells (see below).

Dual immunohistochemical detection of GFP together with other pituitary proteins using green (Alexa488) and red (Cy3) fluorophores demonstrated a novel pattern of cellular co-localization in male rat pituitary glands (Fig. 3). GFP+ cells were initially distinguished into 'large' and 'small' sub-groups by virtue of cytoplasmic extent of GFP protein (see Figs.3A & 3E); cells designated 'large' exhibited a GFP diameter that was 2.5-3.5-fold greater than the nuclear (DAPI staining) diameter and often irregularly shaped, whereas the small GFP+ve cells ranged between 1.5-2-fold greater than the nuclear diameter, and were either uniformly round, or ovoid. Based on the size difference, and our initial observations of large GFP cell co-localization with LHB, cellular co-localization of GFP with pituitary hormone sub-units was quantified by counting co-localization in these two sub-groups separately (Fig. 3; Table 1). As indicated, the large GFP+ cells were extensively LHB+ (Figs. 3A-D) but negative for GH, PRL, ACTH and TSHB (data not shown). Conversely, the small GFP+ cells were largely LHB-negative (Table 1) but extensively expressed GH (Figs. 3E-H) and to a lesser extent PRL (Figs. 3I-L). Interestingly, the GFP/PRL+ cells were often observed with cellular protrusions (Figs. 3I-L). Again, the small GFP+ cells did not express either TSHB (Fig. 3M) or ACTH (Fig. 3N). These results therefore indicate that GFP+ (EGR1) cells in the male rat pituitary are largely made up of gonadotrophs together with somatotrophs and lactotrophs. The cell count data presented here indicates either, the presence of additional GFP/EGR1+ cell-

types or alternatively, inherent errors in the counting procedure that results in under-estimations of the co-localized LHB, GH and PRL populations. Our analysis appears to rule out ACTH and TSHB as possible alternatives. We also investigated three other pituitary cell types: SOX2+ precursor cells (Andoniadou et al, 2013), S100B+ folliculostellate cells (Itakura et al, 2007) and FSH gonadotrophs (Childs et al, 1983). With respect to the first two types, we did not detect co-localization in SOX2+ cells (Fig. 3O) and only rare (1-2/tissue section) GFP co-localization with S100B immunoreactivity in undefined cellular processes (Fig 3P). With respect to FSH, we detected extensive co-localization of FSHB and GFP in the large GFP+ sub-group (Table 1, Figs. 4A-C), but found no evidence of FSHB in small GFP+ cells (Table 1, Figs. 4A-C).

Further studies are required to identify possible alternative, minority EGR1+ cell groups. The present results demonstrate, however, that transcription of the *Egr1* gene is largely restricted to three cell types in the adult rat pituitary gland. The large and small LHB+ cells were often found to be spatially distinct within the pituitary gland; relative to the laterally-concentrated large cells, small cells were generally grouped more medially, adjacent to the neurointermediate lobe (Fig. 4D). The large LHB+ cells were extensively co-localized with GFP; cell counts of LHB/GFP co-localization generated a value (90.7 ± 1.8%, n=6), similar to those for 'Large'GFP/LHB co-localization in Table 1, indicating that these cellular populations are highly similar. Conversely, the small LHB+ cells exhibit only a minor co-localization with GFP (4.0 ± 1.0%, n=6). With respect to large FSHB+ cells, these were extensively co-localized with GFP (47.3±4.8%, n=6) whereas we found no evidence of GFP co-localization in small FSHB+ cells. With respect to GH and PRL cells, GFP was co-localized in minority populations only (GH: 9.0 ± 1.5%; PRL: 3.3 ± 1.0%; n=6).

Discussion

The present study has revealed that *Egr1* is actively, and selectively, transcribed in a major population of male rat gonadotrophs, indicating that this transcription factor is potentially involved in the molecular regulation of gonadotroph function in males. In accordance with previous studies, it is likely that EGR1 acts as a transcriptional regulator of the *Lhb* gene in male rats (Lee et al, 1996; Topilko et al, 1998; Tremblay & Drouin, 1999; Wolfe & Call, 1999). Our results argue that minor expression of EGR1 in male gonadotrophs does not explain the apparent sex-difference in absolute requirement for EGR1 in gonadotroph function (see Tourtellotte et al, 2000). Our study has provided a basis for further investigations of sex differences in pituitary EGR1 function that mirrors a general sexual dimorphism in the regulation of the GnRH-gonadotrophin axis (Colin et al, 1996; as reviewed by Bliss et al, 2010). Because our data also shows, however, that *Egr1* is not actively transcribed in a proportion of male rat gonadotrophs, it may be that only particular aspects of gonadotroph functionality in the male rat pituitary are compromised in the absence of EGR1. In this is indeed the case, then the distinct phenotypes of male *Egr1* knockout mice observed in previous studies (Lee et al, 1996; Topilko et al, 1998), may be explained by differential, strain-dependent, modifier gene influence in the two lines of knockout mice (Tourtellotte et al, 2000).

We have also made the novel observation that *Egr1* is transcribed in significant sub-populations of both somatotrophs and lactotrophs in male rats. These results appear to reveal a major sex-difference. Our previous analyses of *Egr1* expression in female rats showed that approximately 90% of Egr1+ve cells were LHB+ve gonadotrophs (Knight et al, 2000; Man & Carter, 2003). However, the latter two studies were conducted using female rats at the proestrous stage of the oestrous cycle, and currently it is not known whether the described pattern of female EGR1 co-localization is maintained across the

four days of the rat oestrous cycle. The role of EGR1 in the somatotroph/lactotroph populations in male rats is undefined. Clearly, *Egr1* is transcribed in only relatively minority populations in both cases and it may be that this reflects the particular functional dynamics within these sub-populations. In one of the previously established mouse *Egr-1* null-mutants (Topilko et al, 1998), a co-allelic reporter gene was also expressed in some somatotrophs and there was also impaired growth in this particular model. With respect to PRL, it is interesting that GFP/PRL+ cells were often observed with cellular protrusions – these morphological variations may reflect functionally-related changes in cell shape (see Navratil et al, 2007). Our current analysis of 8 pituitary proteins has revealed no evidence for another significant population of EGR1+ve cells in the male anterior pituitary gland; alternative approaches are required to determine whether the current indication of a non-LHB/GH/PRL population simply reflects a counting underestimate that may be related, for example, to the mass of partially overlapping somatotrophs in the tissue sections.

The present study has confirmed a primary role of 5' proximal *Egr1* sequence (1.5kb) in directing cell-specific expression; this accords with our previous studies in brain (Man et al, 2007; Wells et al, 2011), and further indicates that the *Egr1* intronic sequence used in our first generation transgenic model (Man & Carter, 2003) does not have a significant role in spatial or physiological regulation. The relative cellular specificity of *Egr1* transcription is interesting because it is distinct from other inducible transcription factors like *c-Fos*, for example, that is expressed equally in all types of pituitary hormone-producing cells in female rats (Armstrong & Childs, 1997). The specific sequences that direct *Egr1* expression to pituitary sub-populations are interesting for two reasons. First, they could be exploited to control transgene expression in these populations. Second, knowledge of the *trans*-acting factors that interact with these sequences may provide

new insights into cell-specific regulatory mechanisms. Our recent analysis of *cis*-acting sequences within the *Egr1* transgene is consistent with a dominant role for multiple SRE (serum response element) sequences in determining the overall level of transcription (Wells et al, 2011). However, given the ubiquity of SRE-related signaling it is clear that either, other *Egr1* sequence elements must contribute to cellular specificity or alternatively, cell-type selective activation of signaling pathways such as the MAPK pathway (see Man & Carter, 2003) may be involved. One contributing mechanism could be estrogen receptor-linked SRE activation via phosphorylation of the SRF factor, Elk-1 (Duan et al, 2001).

In addition to conferring authentic spatial expression in the rat pituitary gland, our results also demonstrate that the *Egr1* transgene also confers appropriate physiological regulation of expression. This aspect of *Egr1* regulation is also mediated at a transcriptional level, confirming our previous work with an earlier transgenic model (Man & Carter, 2003), but, as noted above, now specifying a primary role for 5' proximal *Egr1* sequence rather than intronic sequence. The demonstrated up-regulation of *Egr1* during proestrous also indicates that *Egr1* 3' UTR sequence (absent from the transgene) does not determine this aspect of regulated expression.

A relatively unrecognized advantage of using GFP and related FPs as a transgene reporter is that cellular filling by this protein can greatly enhance cellular identification and classification (see Wells et al, 2011). Here, we have used this attribute to differentiate between two populations of gonadotrophs. Morphological heterogeneity of gonadotrophs has been described in both female (Childs et al, 1992) and male rats (Jeziorowski et al, 1997) and recent studies have also described functional heterogeneity in male gonadotrophs (Wen et al, 2008). Interestingly, the large GFP+

gonadotrophs were sometimes observed in apparent strings (see Figs. 3A-D), an organization that has also been observed for gonadotrophs in the mouse pituitary (as reviewed by Le Tissier et al, 2012). Currently, the functional distinction between the two populations of male rat gonadotrophs observed in the current study is unknown. Previous work has classified a population of large gonadotrophs as dual LH/FSH-expressing cells (Childs et al, 1983) and our demonstration of FSHB in 50% of large GFP+ cells is consistent with many of these large gonadotrophs being dual expressing cells. The availability of alternative species antibodies may, in future, permit triple co-localization of GFP/LH/FSH and further classification of the cell population identified in our study. Differential sorting of the large fluorescent cells identified in our model (as reviewed by Carter, 2006) could also be used to generate samples for gene expression profiling analysis that could identify molecular classifiers additional to *Egr1*.

We have demonstrated that *Egr1* transcription in male rats is selectively expressed in a significant sub-population of male rat gonadotrophs, allowing us to conclude that a selective association between *Egr1* and *Lhb* is maintained in male as well as female rats, and therefore an absence of *Egr1* in male gonadotrophs does not fully explain sex differences in *Egr1* knockout phenotypes. At the same time, we have made the novel observation that *Egr1* is also actively transcribed in minority populations of somatotrophs and lactotrophs indicating additional pituitary roles for this transcription factor.

Declaration of Interest

There is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author Contributions

PSM, TW and DAC generated the transgenic rat model. PSM and DAC conducted the breeding and experimental analysis. DAC drafted the manuscript which was reviewed and revised by TW and PSM.

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Figure Legends

Fig.1. Sex- and oestrous cycle-dependent expression of the *Egr1-d2EGFP* transgene in adult pituitary glands. A. Chemiluminescence images of immunoblot analyses detecting GFP protein and beta-actin (ACTB) in different protein extracts from individual rats (60µg/lane). A non-transgenic sample (NTG) and purified GFP protein (BD, 1 ng) are included as controls. Note that the transgene-derived protein (d2EGFP) is detected as a doublet that has a slightly greater mass compared with the native GFP due to inclusion of additional (destabilizing) protein sequence. D = diestrus day 1 (metestrus), P = proestrous. Horizontal bars are molecular mass markers. B. Densitometric analysis of GFP protein levels confirmed significantly higher levels of GFP in proestrous, compared with diestrous female rat glands ($P<0.05$, $t = -3.437$, Independent samples t test, n = 3/group. Values Mean ± SEM of fold-difference compared with D. .

Fig.2. Expression of the *Egr1-d2EGFP* transgene is developmentally regulated and recapitulates expression of EGR1 in the adult pituitary. A&B. Direct detection of GFP fluorescence in P5 (A) and P20 (B) pituitary glands showing pan-pituitary expression on P5 that becomes largely restricted to the anterior lobe on P20. C. Representative fluorescence microscopic images of adult male rat pituitary showing immunohistochemically detected GFP (C, green) and EGR1 (D, red). Note both co-localization of GFP and EGR1 (merged images, E & F) and also relative similarity of expression level in a high (arrowheads) and medium (arrows) expressing cell. Blue staining in F is DAPI. Scale bars are 200µm (A), 300µm (B), and 20µm (C-F). AL = anterior lobe; NL = neural lobe.

Fig. 3. *Egr1-d2EGFP* transgene expression in adult male rats is highly co-localized with LHB in large pituitary cells and also co-expressed with GH and PRL in minority

populations of a population of smaller cells. Representative fluorescence microscopic images of adult male rat pituitary showing immunohistochemically detected GFP (green) together with other pituitary proteins (red): LHB (B,C,D), GH (F,G,H), PRL (J,K,L), TSHB (M), ACTH (N), SOX2 (O), S-100β (P). Blue staining in some merged images is DAPI. Arrows show: co-localized GFP and GH in (H); a cellular protrusion in a GFP/PRL co-localized cell in (I-L); minor and rare co-localization of GFP and S-100β immunoreactivity in (P); one example of SOX2/DAPI nuclear co-localization in (O). Scale bars = 20µm.

Fig.4. *Egr1-d2EGFP* transgene expression in adult male rats is co-localized with FSHB in a population of large pituitary cells. A-C. Representative fluorescence microscopic images of adult male rat pituitary showing immunohistochemically detected GFP (green) together with FSHB (red). Blue staining in merged image is DAPI. Note two large cells in the upper part of the images showing co-localized GFP and FSHB. Arrows indicate small cells that are either GFP+/FSHB-ve (left-facing) or GFP-ve/FSHB+ (right-facing). Scale bar = 20µm. D. Distribution of LHB in adult male pituitary gland showing the differential localization of large and small gonadotrophs. Representative fluorescence microscopic image showing immunohistochemically detected LHB in large, laterally localized LHB+ cells (arrows show clusters of cells) and medially localized small LHB+ cells (not labelled) adjacent to the AL/NL border (dashed line). Scale bar = 100µm.