Cartilage-Specific Overexpression of ERRγ Results in Chondrodysplasia and Reduced Chondrocyte Proliferation

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Abstract

While the role of estrogen receptor-related receptor alpha (ERRα) in chondrogenesis has been investigated, the involvement of ERR gamma (ERRγ) has not been determined. To assess the effect of increased ERRγ activity on cartilage development in vivo, we generated two transgenic (Tg) lines overexpressing ERRγ via a chondrocyte-specific promoter; the two lines exhibited ~3 and ~5 fold increased ERRγ protein expression respectively in E14.5 Tg versus wild type (WT) limbs. On postnatal day seven (P7), we observed a 4–10% reduction in the size of the craniofacial, axial and appendicular skeletons in Tg versus WT mice. The reduction in bone length was already present at birth and did not appear to involve bones that are derived via intramembranous bone formation as the bones of the calvaria, clavicle, and the mandible developed normally. Histological analysis of P7 growth plates revealed a reduction in the length of the Tg versus WT growth plate, the majority of which was attributable to a reduced proliferative zone. The reduced proliferative zone paralleled a decrease in the number of Ki67-positive proliferating cells, with no significant change in apoptosis, and was accompanied by large cell-free swaths of cartilage matrix, which extended through multiple zones of the growth plate. Using a bioinformatics approach, we identified known chondrogenesis-associated genes with at least one predicted ERR binding site in their proximal promoters, as well as cell cycle regulators known to be regulated by ERRγ. Of the genes identified, Col2al, Agg, Pth1r, and Cdkn1b (p27) were significantly upregulated, suggesting that ERRγ negatively regulates chondrocyte proliferation and positively regulates matrix synthesis to coordinate growth plate height and organization.

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Introduction

The bones of the axial and appendicular skeleton arise from condensations of chondrogenic cells that lay down a cartilaginous scaffold, which is then remodeled to give rise to the ossified bone. The longitudinal growth of endochondral bones is driven by continued chondrogenesis in the growth plate, which can be divided along the longitudinal axis of the bone into distinct zones comprising resting, proliferating, and post-mitotic chondrocytes, respectively, from the articular surface. The process of chondrogenesis is a highly orchestrated proliferation-differentiation sequence that is regulated by a number of signaling pathways and feedback loops. For example, the homeobox transcription factor, SRY-related high-mobility-group box 9 (SOX9), is the primary determinant of chondrogenesis and is required for the initial commitment of mesenchymal stem cells to the chondrogenic lineage [1]. The hedgehog protein family member, Indian hedgehog (IHH), is also crucial to chondrocyte proliferation and differentiation. It is expressed by prehypertrophic cells and binds to its receptor Patched-1 (PTC-1), which, in turn, activates signaling pathways to promote chondrocyte proliferation [2]. IHH also forms a negative regulatory feedback loop with parathyroid hormone-related protein (PTHrP) to delay chondrocyte hypertrophy and increase the pool of proliferating chondrocytes [3]. On the other hand, the transcription factor RUNX2 promotes the differentiation of chondrocytes from proliferation to hypertrophy [4].

Certain transcription factors belonging to the nuclear hormone receptor family are also involved in chondrocyte differentiation. These include the two estrogen-binding receptors, estrogen receptor alpha and beta (ERRα [NR3A1] and ERRβ [NR3A2] respectively), and recent reports highlight a role for ERα in the fusion or slowing down of growth plate chondrogenesis at puberty in humans and mice. For example, in a cartilage-specific ERRα-deleted mouse, appendicular bones developed normally, but exposure to high levels of estrogen failed to reduce bone length as it did in wild type (WT) mice, indicating that ERα was required for the natural deceleration of bone growth that occurs in mice.
upon sexual maturity [5]. Conversely, a mouse line that expressed a constitutively active form of ER$\alpha$ in cartilage exhibited fewer proliferating cells in the growth plate and reduced bone length [6].

Three orphan nuclear receptor genes related to the ERs comprise the estrogen receptor-related receptor (ERR) family: alpha, beta and gamma (NR3B1, NR3B2, and NR3B3, respectively) [7]. These genes share a high degree of similarity with the ERs, including 67% identity in the DNA-binding domain (DBD), but are unable to bind estrogen [8]. With their similarity in their DBD, it is not surprising that there is considerable cross-talk at the level of gene regulation between the ERs and the ERRs. However, X-ray crystallography studies have clearly shown that, unlike the ERs, the ERRs assume an active state without a ligand bound to the ligand binding domain (LBD) [9,10]. Consistent with the hypothesis that the ERRs are constitutive transcriptional activators, in vitro transcription assays demonstrated that ERR$\alpha$ and ERR$\gamma$ induce expression of target genes without addition of potential ligand to the media [11,12]. ERR$\alpha^{-/-}$ mice display a significant decrease in body mass and ERR$\gamma^{-/-}$ mice are perinatal lethal due to cardiac failure [13,14], phenotypes connected to the roles that both of these isoforms play in energy metabolism.

The role of ERRs in bone and cartilage are also beginning to be investigated, with most data published on ERR$\alpha$ [15]. ERR$\alpha$ is expressed in proliferating chondrocytes in vivo and throughout chondrocyte differentiation in vitro [16]. In addition, it has been shown that ERR$\alpha$ is dysregulated in murine models of inflammatory arthritis [17], as well as in human osteoarthritis [18]. There is no data in the literature describing ERR$\gamma$ in cartilage, and only very little described on its role in bone. Results of an epidemiological study of ERR$\gamma$ polymorphisms in humans indicated a correlation between a subset of ERR$\gamma$ variants and elevated bone mass [19]. In vitro overexpression of ERR$\gamma$ causes a decrease in the expression of bone sialoprotein (B$\beta$) and osteocalcin (Ocn), markers of mature osteoblasts, in the MC3T3 pre-osteoblast cell line [20]. Taken together, these results suggest that ERR$\gamma$ is a negative regulator of osteogenesis. To determine whether ERR$\gamma$ also has a biologically relevant function in cartilage, we have generated transgenic (Tg) mice with a collagen $\alpha$1 (II) (Col2) promoter driving expression of a full length ERR$\gamma$2 (long isoform) transcript (Col2::ERR$\gamma$2FL). We report here that overexpression of ERR$\gamma$2 in a cartilage-specific manner leads to abnormalities in the axial and appendicular skeletons.

### Figure 1. Col2::ERR$\gamma$2 transgenic mouse generation and protein expression analysis.

(A) ERR and ER gene expression (relative to L32) in adult mouse cartilage. Graphs represent mean ± SD from a minimum of 3 independent samples. (B) Schematic of the transgene used to express the long protein isoform, ERR$\gamma$2, in cartilage. Whole mount lacZ staining of a WT and transgenic 14.5 dpc embryo with a close-up of the forelimbs showing robust staining in the developing cartilage. Some nonspecific staining along the neural tube is evident. (C) Western blots and resulting quantification showing the levels of ERR$\gamma$ and $\beta$-ACTIN proteins in 14.5 dpc limbs of the two transgenic lines compared to WT. Graphs represent the mean ± SD from at least three independent Western blots. **p<0.01.

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Results

Overexpression of ERRγ2 Results in Dwarfism

To begin to investigate the putative role of ERRγ in chondrogenesis, we first asked whether ERRγ is expressed in cartilage. We found that ERRγ is expressed in mouse cartilage at levels similar to ERRβ and ERβ, but approximately 50 fold less than ERRα, and 85 fold less than ERRα (Figure 1A). To evaluate the consequences of cartilage-specific overexpression of ERRγ, we generated two independent Tg mouse lines, Line 1 (#6486) and Line 2 (#4094), that express the longer protein isoform of ERRγ under the control of the collagen α1 (II) promoter (Co-12:ERRγ2FL) (Figure 1B). LacZ staining of 14.5dpc embryos revealed distinct staining in the developing craniofacial skeleton and both the axial and appendicular skeletons of Tg mice but not wild type (WT) embryos (Figure 1B). Western blot analysis of 14.5 dpc limbs demonstrated increased expression of ERRγ protein relative to the β-ACTIN control and quantification demonstrated approximately 3-fold overexpression of ERRγ in Line 1, and 5-fold overexpression in Line 2 (Figure 1C). Mice were born in the expected Mendelian ratios and up to at least 8 months of age appeared healthy, suggesting that there is no overt detriment due to the integration or expression of the transgene.

Significant anomalies in all endochondral bones studied and measured were already apparent at birth in Tg versus WT mice (data not shown), but data are reported here only for postnatal day 7 (P7). Measurement of P7 pup weight showed no significant difference in Line 1, but a significant reduction in Line 2 compared to WT mice (Figure 2A). Quantification revealed a small but significant decrease in total body length (data not shown) as well as in the crown-rump length (Figure 2B) in pups of both lines. In P7 whole mount skeletal preparations, Tg mice also exhibited a shortened snout and domed head (Figure 2C) and a significant decrease in skull length, but not width compared to WT mice (Figure 2C). Analysis of specific long bones showed a reduction in the length of the femur and the tibia (Figure 2D) in both lines, as well as the humerus, ulna, radius and scapula (length but not width was affected) (data not shown). No significant differences were observed in Tg versus WT bones that form by intramembranous ossification, e.g., the mandible and the clavicle (data not shown). To rule out the possibility that the phenotypes we observed were due to the sex of the pups, we genotyped them for gender, and found that the phenotypes we describe occur in both male and female mice. Taken together, the data indicate that targeted ERRγ overexpression in cartilage results in mild dwarfism.

Overexpression of ERRγ Impairs Chondrocyte Proliferation, Differentiation-maturation, Cartilage Matrix Production and Growth Plate Organization

Quantification of P7 proximal humerus, distal femur, and proximal tibia revealed trends or significant decreases in total growth plate height as well as the proliferative and hypertrophic zones in Tg versus WT mice (Figure 3A and B). The most dramatic decrease was in the proliferative zone, which displayed a 22% height reduction in the transgenic versus WT mice, whereas hypertrophic zone changes were less pronounced and not detectable in all bones (Figure 3B). The quantification of zone heights in the Tg mice was complicated by 2 factors: a smaller and disorganized proliferative zone, and the presence of acellular swaths of cartilage matrix that often spanned the resting, proliferative and hypertrophic zones (Figure 3A). Although we observed 2 WT samples containing acellular swaths, such areas were much smaller and less pronounced than those in Tg samples.

To determine the basis of the reduction in growth plate zone heights, we quantified the number of proliferating cells by immunostaining for the proliferation marker, Ki67 (Figure 4A). No significant difference was observed in resting zone chondrocytes, but a 50% decrease in Ki67-positive cells was seen in the Tg compared to WT proliferative zone (Figure 4B). Since acellular masses of matrix have been reported previously in other genetically-modified mice, including ones with unusually wide or generally disorganized growth plates in which hypoxic cell death occurs [1], we next performed a TUNEL assay. No significant difference in TUNEL staining was observed in either the hypertrophic or proliferative zones of Tg versus WT growth plates (quantified for Line 2; Figure 4C). RT-qPCR also revealed no differences in expression of the apoptosis-associated markers, Bax and Bcl2, in RNA isolated from the growth plate of WT versus Tg mice (Figure 4D). The data suggest that the reduced length of the proliferative zone is a consequence of decreased chondrocyte proliferation, whereas that of the hypertrophic zone may be due to a disruption in chondrocyte differentiation, a delay in chondrocyte maturation, or secondary effects to the reduction in proliferation.

To investigate the molecular basis of changes observed in the growth plate as a consequence of overexpression of ERRγ2, we next used an in silico database search to screen for putative ERR binding sites in the region spanning 10 Kb upstream to 5 Kb downstream of the transcriptional start site in a variety of genes known to be involved in chondrogenesis. The genes that met both criteria are listed in Table 1 and included transcription factors (Sox9, Sox6, Altf, and Runx2), extracellular signaling molecules (Bmp, Bmp7, and Pthrp), hormone and growth factor receptors (Pth1r and Fgfr3), and cartilage matrix proteins (Col2, Col2a1, Col10, Col11a1, and Agg). Because we observed a reduction in proliferation within the proliferative zone of transgenic animals, and because ERRγ has been shown to suppress proliferation [21], we also assessed the expression of cell cycle regulators cyclin D1 (Ccnd1), and cyclin dependant kinase inhibitor 1a and 1b (Cdkn1a and Cdkn1b). RT-qPCR revealed a significant increase in Pth1r (Figure 5A), but no differences in the transcription factors tested (Figure 5B). We also observed an increase in cartilage matrix proteins Col2 and Agg (Figure 5C) and the cell cycle regulator Cdkn1b (p27) (Figure 5D) in Line 2 (higher overexpresser). This suggests that overexpression of ERRγ decreases chondrocyte proliferation through regulation of Cdkn1b, impacts growth plate organization, matrix synthesis as evidenced by upregulation of Col2 and Agg, and affects chondrocyte maturation through upregulation of Pth1r.

Discussion

We report here that overexpression of ERRγ2 in chondrocytes results in decreased axial and appendicular skeleton size and disruption of growth plate height and organization. This phenotype was manifested already in newborn mice, indicating an effect on embryonic skeletal development that persisted postnatally, and included changes in chondrocyte proliferation, differentiation, and maturation, as well as matrix production, suggesting a role for ERRγ in chondrogenesis.

In the two independent Col2::ERRγ2FL mouse lines analyzed, ERRγ2 is overexpressed at moderate levels (3 to 5 fold higher levels than endogenous ERRγ expression in WT littermates) and the resultant skeletal phenotype and concomitant changes in gene expression observed are relatively subtle and ERRγ dose-dependent. It therefore seems likely that the phenotype seen in Col2::ERRγ2FL mice is a direct consequence of modulation of ERRγ transcriptional activity rather than from disruption of the
transcriptional machinery. Nevertheless, it remains possible that the effects of ERRγ2 overexpression are indirect, e.g., resulting from an imbalance in the ratio of the ERRγ1/ERRγ2 protein isoforms and sequestration of required transcription cofactors (see also below). Thus, further studies in ERRγ knockout mice, in particular chondrocyte-specific ERRγ knockout mice to circumvent the perinatal lethality seen in global ERRγ knockout mice ([14] and Cardelli and Aubin, unpublished data), and in Col2:ERRγ1 overexpressing mouse lines, are of interest.

The most pronounced effect observed in the Tg growth plate was the reduction in height of the proliferative zone, with a 22% reduction in proliferative zone length and a 30% decrease in the percentage of proliferative Tg compared to WT cells. Despite the fact that CcnD1 contains 2 putative ERREs in its regulatory region (Table 1), we did not observe any difference in CcnD1 expression in WT versus Tg 14.5 dpc limbs. However, we observed increased expression of the cyclin-dependent kinase inhibitor Cdkn1b in Tg limbs, suggesting regulation of chondrocyte proliferation through regulation of this kinase inhibitor, a possibility consistent with data showing that ERRγ suppresses S-phase progression in an in vitro model of prostate cancer through transactivation of Cdkn1a and Cdkn1b [21]. A scan of the Cdkn1b regulatory region reveals 3 putative ERRE binding motifs at positions −5077, −2625, and

Figure 2. Skeletal analysis of P7 animals. (A) Body weight measured in P7 pups from WT (n = 19), Line 1 (n = 11) and Line 2 (n = 9) animals. (B) Crown-rump length measurement showed reduced axial skeleton. Measurements were taken from the snout to the base of the tail from WT (n = 24), Line 1 (n = 16) and Line 2 (n = 13) pups. (C) Close-up photographs of the skulls from WT and Tg animals, and resulting quantification showing reduced length, but not width, in Tg compared to WT animals, WT (n = 6) and Line 1 (n = 11) pups. (D) Alcian blue/Alizarin red double stain of WT and Line 2 hindlimbs. Quantification shows reduced bone length and mineralized component of the axial skeleton in Tg compared to WT animals. Total femur and tibia length were measured as well as the length of the mineralized portion of the bone as demarcated by the Alizarin red staining for WT (n = 22), Line 1 (n = 14), Line 2 (n = 14). Graphs represent the mean ± SD *p ≤ 0.05, **p ≤ 0.01, ***p ≤ 0.001.

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−2618, suggesting potential direct regulation, however we cannot rule out a novel, indirect protein interaction.

In addition to the changes in cell cycle regulators and proliferation, we observed slightly reduced hypertrophic zone length in some bones, marked disorganization of the proliferative and hypertrophic zones, and acellular swaths that spanned a large portion of the Tg growth plate, which were not due to an increase in apoptosis. Moreover, Col2, Agg, and Pth1r expression were increased in 14.5 dpc Col2ːERRγFL mice. Taken together, the data suggest that ERRγ plays a role in coordinating chondrocyte proliferation-differentiation-matrix synthesis, but whether the changes are directly and causally related remain to be determined. Nevertheless, increased COL2, a cartilage extracellular matrix (ECM) protein that binds to integrins and activates signaling pathways essential to chondrocyte proliferation, may contribute to both acellular swaths of cartilage and decreased chondrocyte proliferation, via a negative feedback loop between abundant matrix production and proliferation. For example, it has been shown in chondrocytes from mouse ribcage that lack of β1 integrin results in reduced chondrocyte motility and COL2 adhesion, as well as reduced CcnD1, and increased Cdkn1a expression, resulting in decreased chondrocyte proliferation [22]. Further, it has been shown that ERRα is involved in osteoclast migration and adhesion, in part through regulation of β3 integrin [23]. It is necessary to determine whether integrin expression and/or

Figure 3. Growth plate analysis of P7 pups. (A) Growth plates from WT and Line 2 femurs showing the reduced proliferative zone and the acellular swath observed in the transgenic animals. (B) Analysis of total, resting, proliferating, and hypertrophic growth plate heights from proximal humerus (PH), distal femur (DF) or proximal tibia (PT). The largest decrease in growth plate height was observed in the proliferating zones of Tg animals compared to WT. *p≤0.05, **p≤0.01, ***p≤0.001 RZ, Resting Zone; PZ, Proliferative Zone; HZ, Hypertrophic Zone; AS, Acellular swath. doi:10.1371/journal.pone.0081511.g003
Figure 4. Decreased proliferation, but not apoptosis, is responsible for reduction of growth plate height. (A) Hoechst labeling and immunofluorescence of proximal humerus growth plate from WT and Line 2 samples, showing Ki67 positive cells in the proliferating zone (PZ) (red boxes), and resting zone (RZ) (white box). 2° Ab is a section stained with secondary antibody only and Hoechst. Lower panel shows the proliferative
chondrocyte adhesion and motility are affected in the ERRγ-overexpressing mice.

Whether Col2 is regulated directly by ERRγ or indirectly through interaction with a factor upstream of Col2 remains to be determined. It has been shown that overexpression of NKK3.2 in vitro increases Col2 expression in a SOX9-independent manner.

Table 1. Results of in silico search for putative ERRE sites in known regulators of growth plate chondrocytes (RZ = resting zone; PZ = proliferative zone; PHZ = pre-hypertrophic zone; HZ = hypertrophic zone).

| Gene  | Expression | Function        | Putative ERRE Position (bp) |
|-------|------------|-----------------|-----------------------------|
| AIF4  | PZ & PHZ   | Proliferation   | ACAAGGACA −7522             |
|       |            |                 | TTAAGGTCA −6669             |
|       |            |                 | ACAAGGACA −3214             |
|       |            |                 | CAAAGGTCA −606              |
|       |            |                 | CAAAGGTCA +3245             |
|       |            |                 | CTAAGGTCA +4796             |
| Bmp7  | PHZ        | Proliferation & | TCTAGGTCA −4858             |
|       |            | Differentiation | TAAAGGTCA −4235             |
|       |            |                 | CAAAGGTCA +1692             |
|       |            |                 | CAAAGGTCA +3245             |
| CcnD1 | RZ, PZ, PHZ, HZ | Proliferation | TCTAGGTCA −4175             |
|       |            |                 | TGAAGGTGA +1117             |
| Fgrf3 | PHZ, PHZ, HZ | Proliferation & | TGAAGGTCA −6661             |
|       |            | Differentiation | TAAAGGTCA +4663             |
| Ihh   | PHZ        | Proliferation & | TAAAGGTCA −5988             |
|       |            | Differentiation | TCAAGGTCA −2716             |
|       |            |                 | TCAAGGTCA +1353             |
|       |            |                 | CAAAGGTCA +4046             |
| Pth1r | PZ, PHZ    | Proliferation & | CAAAGGTCA −9433             |
|       |            | Differentiation | CAAAGGTCA +2698             |
| Runx2 | PHZ, HZ    | Differentiation | CAAAGGTCA −6895             |
|       |            |                 | CAAAGGTCA −3156             |
| Sox6  | RZ, PZ, PHZ | Proliferation & | TCAAGGTCA −6264             |
|       |            | Differentiation | ATGGAGTCG −2802             |
| Sox9  | RZ, PZ, PHZ | Proliferation & | TCAAGGTCA −978              |
|       |            | Differentiation | CAAAGGTCA −8797             |
|       |            |                 | CAAAGGTCA −2966             |
| Pthrp | RZ, PZ, PHZ | Proliferation & | TCAAGGTCA −2728             |
|       |            | Differentiation | CAAAGGTCA −978              |
| Col2  | RZ, PZ, PHZ | Cell-matrix     | TCAAGGTCA −8714             |
|       |            |                 | GATAGGTCA −7002             |
|       |            |                 | GTAAGGTCA −2325             |

Position is relative to the start site. doi:10.1371/journal.pone.0081511.t001

by directly binding to a 48 bp chondrocyte-specific enhancer in the Col2 regulatory region [24]. Scanning the regulatory region using a core ‘AGGTCA’ sequence reveals 3 putative ERREs through which ERRγ could directly control Col2 expression. Alternatively, ERRγ may regulate Col2 expression through the recruitment of cofactors, such as PGC1-α and CBP/P300, as has been shown in SOX9-dependent regulation of Col2 [25,26]. Intriguingly, one of the ERREs in the Col2 regulatory region is near the SOX9 binding site, supporting the hypothesis of a larger transcriptional regulatory complex.

Analyses of several genetically-engineered mouse models have revealed the importance of FGFR2 [29,30] and IHH [3,30,31] signaling in chondrocyte proliferation and differentiation. Recently, it has been reported that several transcription factors regulate the transcription of Ihh, including RUNX2 [32], ATF4 [33], and MSX2 [34]. The phenotype we observed in the ERRγ-overexpressing mice did not result in transcriptional changes in Ihh or Fgfr3, suggesting either a regulatory mechanism involving a transcriptional complex with one of the above mentioned transcription factors or regulation independent of IHH or FGFR3.

It will also be important to further analyze how ERRγ regulates chondrocyte maturation and hypertrophy, as no significant changes were detected in the hypertrophy markers assessed, such as Runx2, or its target gene Col10. While this may reflect simply the small content of hypertrophic cells in the 14.5 dpc samples utilized, it is worth noting that we also detected no differences in Col10 expression in 17.5 dpc or P7 bone samples (data not shown). This suggests that ERRγ may have only a small or secondary role in chondrocyte hypertrophy. Alternatively, the modest difference we observe in the Tg hypertrophic zone height may be too small to quantitatively detect significant differences in hypertrophic marker gene expression. As mentioned, the presence of the acellular region in our mouse model, additional studies on the growth plate of early embryonic stages [35]. By contrast, transgenic mice that constitutively express Pth1r display a severe delay in the endochondral process, including a reduced zone of hypertrophy [36]. Although we did not observe any differences in hypertrophic gene expression in our mouse model, additional studies on the growth plate of early embryonic mice are needed to elucidate the basis of hypertrophic zone anomalies.

In addition to the above factors, estrogen and expression of soluble and membrane-bound estrogen receptors (ERα and GPR30, respectively) have also been implicated in regulating growth plate chondrogenesis. Mice expressing Col2 promoter-driven ERα have reduced proliferation and differentiation, and subsequent dwarfism [6], while mice with cartilage-specific
inactivation of ERα exhibit prolonged longitudinal bone growth [5]. Taken together, the evidence suggests that not only ERα, but also ERRγ are negative regulators of chondrocyte proliferation and differentiation, which appear to be opposite to the function of ERRα [16]. Further, ERRγ is able to form heterodimers with ERRγ [37] and ERα [38], suggesting that regulation of chondrocyte proliferation and differentiation may require a carefully controlled balance of the nuclear receptors. It remains to be elucidated if any interaction occurs between ERRγ and ERRα during chondrogenesis. It is also possible that the high ERRγ2 expression in our model may turn ERR transcriptional activation into repression. It was demonstrated that ERRα and ERRγ can independently activate an ERR-driven promoter reporter, but when heterodimers of ERRα/γ were formed, the same reporter was suppressed [37].

Figure 5. Expression profile of putative target genes. Genes are grouped as extracellular growth factors (A), transcription factors (B), ECM proteins (C), and cell cycle regulators (D). Of the genes tested, we observed increased Pth1r, Col2α1, Agg, and Cdkn1b expression in 14.5 dpc limbs of Tg compared to WT animals. Expression by RT-qPCR of putative target genes was normalized to the expression of L32. WT (n = 7), Line 2 (n = 9), *p≤0.05. doi:10.1371/journal.pone.0081511.g005
In summary, overexpression of ERRγ2 in a cartilage-specific manner leads to dose-dependent abnormalities in the axial and appendicular skeletons due to alterations in Col2a1 expression and chondrocyte proliferation as well as differentiation-maturation-matrix synthesis. Work is ongoing to characterize further the mechanism by which ERRγ exerts its actions in the developing growth plate.

Materials and Methods

Ethics Statement

All experimental procedures were performed in accordance with protocols approved by the Canadian Council on Animal Care and the University of Toronto Faculty of Medicine and Pharmacy Animal Care Committee.

Construction of pCol2a1mERRγ2 and Generation of Transgenic Mice

We made a generic transgene vector containing the mouse Col2a1 promoter (gift from B. de Crombrugghe) and flanking intron [39] fused to a splice acceptor site [40] with stop codons in all reading frames, an extensive multiple cloning site (MCS), a Polio internal ribosome entry site (gift from P.A. Greer), a nuclear localized β-galactosidase [41] and the protamine minigene pA [42]. During the construction of this vector, silent mutations were introduced to abolish restriction sites within the transgene so that the MCS could be expanded for future cloning of cDNAs. The transgene is flanked by NotI restriction sites. The longer 458 base pairs amino acid open reading frame for ERRγ2 (NM_001243792) was PCR amplified from mouse muscle cDNA with primers containing restriction enzymes to facilitate cloning. All vectors were verified by sequencing. Transgenic lines carrying the pCol2a1mERRγ2 construct were generated by pronuclear injection, as described previously [43]. Hemizygous founders were screened for transgene expression by performing PCR on DNA isolated from tail clips taken from the F1 generation of progeny (Figure 1). To determine sex of the P0 or P7 pups, we performed PCR targeting the sex-determining region Y (Sry) (Table 2).

LacZ Stain for Detection of Transgene

Embryos of 14.5 dpc were dissected, and processed for LacZ detection as previously described [44].

Gene Expression Analysis

Embryonic tissues and adult articular knee cartilage were harvested using an Ultra Turrax T25 homogenizer, while postnatal tissues were manually ground with a mortar and pestle under liquid nitrogen. The RNA was extracted using TRIzol (Invitrogen), precipitated with isopropanol, and resuspended in 50–200 μL of DEPC dH2O. To remove potential contaminating DNA, RNA samples were subjected to DNase treatment, using an Ambion Turbo DNA-Free kit (Ambion), according to the manufacturer’s directions. Three μg of DNased RNA were reverse transcribed using Superscript II Reverse Transcriptase (Invitrogen), according to the manufacturer’s directions. All primers were designed with intron inclusion in corresponding genomic DNA, and are common to all potential transcript variants (Table 2).

Western Blotting

Dissected limbs in PBS were homogenized (Ultra Turrax T25), followed by lysis in RIPA buffer (50 mM Tris HCl pH 8, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate and 0.1% sodium dodecyl sulphate) with added protease inhibitors. Protein samples were quantified using the Bio-Rad DC Protein Assay kit, following the manufacturer’s instructions. Thirty μg of each sample was run in a 10% SDS-PAGE gel, transferred to polyvinylidene fluoride (PVDF) membrane, followed by blocking in 5% milk-TBS-T for 30 minutes at room temperature. Immunodetection was carried out using a rabbit polyclonal anti-ERRγ antibody (H38x, Santa Cruz Biotechnology Inc.) diluted 1:5000 in blocking buffer, or rabbit anti-β-ACTIN antibody (Abcam, Cambridge, MA) diluted 1:2000 in blocking buffer, followed by incubation with a goat anti-rabbit IgG, conjugated to horse radish peroxidase (HRP; Santa Cruz Biotechnology Inc.) diluted 1:5000–1:8000 in blocking buffer. The HRP was visualized using the Amersham ECL Western Blotting Detection kit (GE Healthcare), as per the manufacturer’s instructions. The autoradiographic films from both ERRγ and β-ACTIN detection were scanned and the density and size of the bands were quantified using Image Lab software (Bio-Rad). The ERRγ band was normalized to the β-ACTIN band to assess expression we see to the ERRγ isoform. Whole Mount Skeletal Staining

P0 and P7 animals were dissected, eviscerated, and fixed in 95% ethanol overnight or up to two weeks, and then processed for whole mount skeletal staining as previously described [45]. When samples were fully cleared, skeletons were dissected and photographed in a Petri dish containing 100% glycerol, using a Nikon microscope.
Coolpix P5100 digital camera affixed to a dissecting scope. The images were then quantified in Image J by taking linear measurements of individual skeletal components.

**Histological and Immunofluorescence Analysis**

The left limbs taken from each skeleton analyzed by whole mount staining were fixed in 4% paraformaldehyde for 24 hours, transferred to PBS for 2–3 days and then decalcified (10% EDTA, 0.1 M Tris pH 7.4) for 15 days. Once decalcified, limbs were serially dehydrated with one 20 minute wash in 30% ethanol, three 20 minute washes in 50% ethanol and a final wash and storage in 70% ethanol. Samples were paraffin-embedded, sectioned (5 μm), and stained (hematoxylin and eosin, and Safranin-O) at the Toronto Centre for Phenogenomics (TCP). The Safranin-O-stained slides were photographed under a Nikon Eclipse TS100 fluorescence microscope, at magnifications of 40X and 100X. In the 40X micrographs, measurements were taken from the absolute edge of Safranin-O staining before the articular surface, down to the last hypertrophic chondrocyte before the primary spongiosa. Three such measurements were taken at separate axes along the bone, with the average representing the total growth plate height. Using the 100X micrographs, measurements were taken from the first flattened chondrocyte, which appeared in a distinct column of three or more cells, down to the last hypertrophic chondrocyte, and then the subset of that distance that contained only hypertrophic chondrocytes was taken. This was repeated at five points along the width of each growth plate, and the averages were used to calculate the specific heights of the resting, proliferative and hypertrophic zones. For each animal, growth plates were assessed from the proximal humerus, distal femur and proximal tibia.

To immunodetect Ki67, a common proliferation marker [42], sections were deparaffinized and rehydrated in ethanol washes, followed by antigen retrieval in boiling citrate buffer (10 mM citric acid, 0.05% Tween-20, pH 6) for up to 20 minutes. The slides were blocked in normal goat serum (Invitrogen) for 30 minutes at room temperature, washed, incubated with rabbit polyclonal anti-Ki67 antibody (diluted 1:25 in blocking buffer) for 1 hour at room temperature, washed, then incubated with goat anti-rabbit Alexa-594-conjugated secondary antibody (Invitrogen, diluted 1:50 in blocking buffer) for 30 minutes at room temperature. The samples were counterstained with Hoechst nuclear stain.

Growth plate sections of the proximal humerus were viewed and imaged using a Bioquant Osteo imager with Photofluor II fluorescence excitation light, a triple Chroma filter, and Bioquant Osteo 2012. Ki67 positive and negative cells within the proliferative and resting zones were counted for TUNEL analysis and normalized to methyl green stained nuclei.

**TUNEL Assay**

Growth plate sections of the proximal humerus were processed as described above, before using the FragEL DNA fragmentation detection kit (Calbiochem), as per the manufacturers instructions, and counterstained with methyl green. Images were sectioned at 16X magnification, using a Nikon Eclipse TS100 fluorescence microscope. Images were analyzed using ImageJ. Cells within the proliferative and hypertrophic zones were counted for TUNEL analysis and normalized to methyl green stained nuclei.

**Analytical Techniques**

**Expression Analysis of Putative Target Genes**

A list of putative target genes was constructed by in silico analysis of the regulatory regions of factors known to be involved in the chondrocyte differentiation process and that contained at least one ERR binding site (ERRRE) in the region from −10 Kb to +5 Kb of their annotated transcription start site. The search used the ERRRE consensus sequence TCAAGGTCA and 20 additional sequence variants that had been identified in the literature [46]. The search was performed using the freely available Transcriptional Regulatory Element Database (TRED) (http://rulai.cshl.edu/cgi-bin/TRED/tred.cgi?process=home), which retrieves genomic sequences from the current Ensembl build of the mouse genome. The results from this search are shown in Table 1, along with the primers used to look for differences in gene expression in Table 2. The primers were chosen to pick up all of the known transcript variants and include at least one intron in the corresponding genomic DNA.

The reactions were performed in triplicate on a 96-well plate in a BioRad MyIQ iCycler, for 50 cycles with an annealing temperature of 59°C. The amplification data was uploaded into the PCR miner program (http://www.ewindup.info/miner/version2/) to obtain the Ct and reaction efficiency values. The relative expression levels of the target gene were normalized to the L32 internal control expression.

**Statistical Analysis**

All data were analyzed using Graphpad Prism 4.0 software. When three data sets were analysed, ANOVA was used first to determine significance, followed by Student’s T-Test. All the graphs are plotted as the mean ± standard deviation and the p values listed are for the comparison to the WT values. Graphs were constructed using Microsoft Excel 2003 software.

**Mouse Gene Nomenclature**

We followed the mouse nomenclature guide as stated on the Mouse Genome Informatics web page (http://www.informatics.jax.org/mgihome/nomen/short_gene.shtml). Thus, mouse genes are written with first letter capitalized followed by small letters, all italicized, while proteins are written all capitalized and without italics.

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**Author Contributions**

Conceived and designed the experiments: MC RAZ JFB KPM T-CT KT JEA. Performed the experiments: MC RAZ JFB KPM T-CT. Analyzed the data: MC RAZ JFB KPM T-CT KT JEA. Contributed reagents/materials/analysis tools: MC RAZ JFB KPM T-CT KT JEA. Wrote the paper: MC RAZ JFB KPM. Data interpretation: MC RAZ JFB KPM. T-CT KT JEA. Revising manuscript content and approving final version of manuscript: MC RAZ JFB KPM T-CT KT JEA. Responsible for the integrity of the data analysis: MC RAZ JFB KPM T-CT KT JEA.
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