Introduction

Disruption in the normal adolescent growth spurt can cause the spinal deformities that result in idiopathic scoliosis. It is defined by the presence of lateral deformity of the spine, with otherwise normal vertebrate bodies and without other diagnoses. Due to its prevalence of 2%-3% in school-aged children it poses a considerable health burden in the pediatric population. In general, spinal curves can be classified into congenital, neuromuscular, and the idiopathic forms [1,2]. Congenital forms of scoliosis involve structural malformations of the spine that are visible on radiographs and include segmental abnormalities such as hemivertebrae, wedge-shaped vertebral bodies, and vertebral fusions and bars. In contrast to most idiopathic forms of scoliosis, congenital forms are resistant to correction and frequently progress to cause severe deformation, thus pose the most clinical problems [3]. In line with the segmental patterning that leads to the formation of the spine, four mutations associated with congenital scoliosis have been found in genes associated with the human segmentation clock mechanism (DLL3, MESP2, LFNG, HES7) [4]. Genome-wide association studies have been performed for families with idiopathic scoliosis and have identified polymorphisms in one gene (CHD7) that regulates multiple genetic pathways [5]. This implies that variations in other genes responsible for rare disorders may likewise contribute to idiopathic scoliosis. This notion has been contended before [6] arguing that so-called “idiopathic” scoliosis may be the result of sub-clinical lower motor neuron disorder. Histochemical studies of the thoracic part of the erector spinae muscles in scoliosis have shown consistently a changed fiber structure on the convex versus the concave side of the spine. Thus, this deviation in adult onset idiopathic scoliosis also may constitute one of the primary factors in the pathogenesis of the spinal curvature [7-9], but controversies still exist [10]. In any case, the formation of contractile myofibrils requires the ordered stepwise onset of expression of muscle specific proteins. Any defects in the expression patterns of muscle-specific genes may underlie muscle disorders [11] and, consequently, congenital or idiopathic disorders of the human skeletal apparatus including scoliosis. Changes in mRNA levels have been shown to be the primary genetic defects in muscular dystrophies, including mRNA for embryonic myosin heavy chain, α-cardiac actin, versican, acetylcholine receptor α-1, thrombospondin 4 and others [12]. Further, a heterozygous missense mutation in the MEGF10 gene was found to impair the regeneration of adult muscle in response to injury or disease and leads to myopathy and scoliosis [13]. Minor defects in muscle-specific genes might thus instigate adolescent-onset idiopathic scoliosis due to altered responses to a changed growth factor environment during...
the critical period of rapid growth. Thus, it was of interest to stimulate myocytes in in vitro cultures by various growth factors and highlight their effects on the cells by assessing the expression of keygenes.

Materials and Methods

Materials

All chemicals and cell lines were ordered from the company PromoCell unless it is declared otherwise. Reagents mentioned in the chapter ‘Materials for reverse transcription’ were from the company Roche Diagnostics. The Mastermix and primer mentioned in the chapter ‘Materials for real-time PCR’ were ordered from Applied Biosystems.

Cell Culture

The cryopreserved human skeletal muscle cell lines (C-12530) was obtained from PromoCell, Heidelberg, Germany). Cells derived from skeletal muscle tissue from different locations. The vial contained 500,000 cells/ml.

Myocyte Basal Medium (C-22270) (PromoCell) with a supplement pack (C-39270), containing all supplements necessary for the optimal growth of human myocytes according to the manufacturer’s protocol, Pen/Strep/Fungizone (PromoCell, C-42020), and 10% foetal bovine serum (PAA, A15-101) was used for culturing the cells. Cells were cultured at 37°C and 5% CO₂. Daily visual inspection of the cells during expansion indicated mostly undifferentiated cells. Myoblasts were not differentiated to myotubes. Cells were cryopreserved at passage 2 and passed another time prior to the experiments. Growth factors, fFGF (P0291, Sigma-Aldrich, St. Louis, Missouri) and IGF (SRP3069, Sigma-Aldrich) were diluted as prescribed. Experiments were performed during the exponential growth phase, with approximately 1e7 cells per 75 cm² flask. For subcultivating the cells, Trypsin PBS (C-41050) was used for detaching the cells from the bottom of the culture flask.

RNA isolation

Cells were treated with IGF1 or FGF2 (10 ng/ml) for 24 hours. Control cells were kept in a medium without added growth factors. After incubation, cell plates (two plates for all experiments) were rinsed with Trizol™ (Life Technologies, Darmstadt, Germany) for immediate mRNA extraction after the culture medium was removed and mRNA was purified according to the manufacturer’s instructions. mRNA was reverse transcribed directly and the CDNA was stored at-70°C until the point of analysis. This protocol ensured the highest possible quality of mRNA, preserving the mRNA within seconds from nuclease digest. Random primers, deoxynucleotidetriphosphates, protector RNase inhibitor and reverse transcriptase were obtained from Roche (Basel, Switzerland). RT-PCR was performed using Roche Fast Start DNA Master HybProbe (Roche) and Taqman primers (for GAPDH, CHD7, HDAC5, ACTA1, Lef1, WNT5A, COL1A1, COL2A1, ACAN, FGFI and VCAN) obtained from Life Technologies (Newton Drive, Carlsbad, USA). RT-PCR was performed on an ABI Prims 7000 detection system (Life Technologies). 6-Carboxylfluorescein (FAM) fluorescence was used as readout. The amplification blots were checked visually and the baseline was set manually. Every RTPCR reaction was run in triplicate for every cDNA (Table 1). Expression levels of the various genes are shown as means plus standard deviation of the triplicate qPCR measurements.

Results

For the stimulation of muscle cells, two different growth factors, FGF2 and IGF1, were chosen. FGF2 is involved in proliferation of muscle cells and IGF1 induces maturation and enlargement of skeletal muscle cells, and it also stimulates hypertrophy of myoblasts. Cultured myoblasts were analyzed for the expression of seven genes with possible links to scoliosis or the segmentation of the spine: chromomodain helicase DNA binding protein 7 (CHD7), histone deacetylase 5 (HDAC5), actin alpha 1 skeletal muscle (ACTA1), aggrecan (ACAN), collagen type I alpha 1 (COL1A1), lymphoid enhancer-binding factor 1 (LEF1), and wingless-type MMTV integration site family, member 5A (WNT5A). PCRs in triplicates were performed with these genes.

The expression of the housekeeping gene GAPDH was used as a reference for calculating the relative gene expressions. The difference in the threshold value (Ct) between the stimulated cDNA for a certain gene and GAPDH - ∆∆Ct1-and the difference between the unstimulated cDNA for the same gene and GAPDH - ∆∆Ct2-were calculated. The ∆∆Ct is the difference between the two ∆Ct. Results were attained by the formula 2^(-ΔΔCt). All experiments were performed at least two times.

Two patterns of response to the growth factors were observed: Five genes (CHD7, HDAC5, COL1A1, ACAN, LEF1) (Figure 1) were stimulated in their level of expression by IGF-1 with lesser or no effects of FGF2, and one gene (WNT5) (Figure 2) was even down regulated by the addition of FGF2. Only ACTA1 showed an increased expression level that was augmented higher by FGF2 than by IGF-1 (Table 1).

Table 1: Influence of growth factors on the expression of the genes shown in relation to GAPDH Values are the results of one representative experiment on the effects of the growth factors FGF2 and IGF-1 on gene expression of myocytes in in vitro culture. After mRNA extraction, three independent PCR experiments were performed; data show the mean and the upper and lower boundaries in relation to GAPDH expression.

| Growth factor | gene   | Fold difference | Lower boundary | Upper boundary |
|---------------|--------|-----------------|----------------|---------------|
| FGF           | CHD7   | 26.253          | 10.385         | 66.368        |
| IGF           | CHD7   | 47.672          | 18.908         | 120.193       |
| FGF           | HDAC5  | 12.813          | 0.5072         | 32.366        |
| IGF           | HDAC5  | 20.096          | 0.7906         | 51.083        |
| FGF           | ACTA1  | 60.514          | 23.159         | 15.812        |
| IGF           | ACTA1  | 56.218          | 21.949         | 143.992       |
| FGF           | COL1A1 | 0.9205          | 0.3645         | 23.247        |
| IGF           | COL1A1 | 21.138          | 0.8359         | 53.452        |
| FGF           | ACAN   | 21.685          | 0.84           | 55.979        |
| IGF           | ACAN   | 33.048          | 12.627         | 86.493        |
| FGF           | Lef1   | 10.157          | 0.6085         | 16.953        |
| IGF           | Lef1   | 20.043          | 11.281         | 35.611        |
| FGF           | WNT5A  | 0.5762          | 0.3452         | 0.9617        |
| IGF           | WNT5A  | 11.516          | 0.6608         | 2.007         |

Citation: Huber K, Kraupa C, Kluger R and Krugluger W. Gene Expression Profiles Induced by Growth Factors in In Vitro Cultured Myocytes. SM Musculoskelet Disord. 2018; 3(2): 1031. https://dx.doi.org/10.36876/smmd.1031
mechanisms for embryonic, fetal, postnatal, and adult regenerative myogenesis are likely to be similar [14,15], cultured myoblasts were analyzed for the expression of seven genes with possible link to scoliosis or the segmentation of the spine: chromodomains helicase DNA binding protein 7 (CHD7), Histone Deacetylase 5 (HDAC5), Actin Alpha 1 Skeletal Muscle (ACTA1), aggrecan (ACAN), Collagen Type 1 Alpha 1 (COL1A1), Lymphoid Enhancer-Binding Factor 1 (LEF1), and wingless-type MMTV integration site family, member 5A (WNT5A).

The first four genes were chosen, because of known polymorphisms which are partly associated with scoliosis [13]. Collagen type 1 alpha 1 is necessary to provide a passive elastic substrate to support myofibres and facilitate the transmission of force for skeletal muscle [16]. WNT5A and LEF1 are part of the Wnt-signaling pathway and therefore we were interested whether they are also active in muscle cells as most transcriptional endpoints of this pathway are cell type specific [17].

For the stimulation of muscle cells, two different growth factors, FGF2 and IGF1, were chosen. FGF2 is involved in proliferation of muscle cells [18,19] and IGF1 induces maturation and enlargement of skeletal muscle cells, and it also stimulates hypertrophy of myofibers [20,21]. In this first analysis, we were interested whether these growth factors influence at all the panel of genes chosen. By this, we attempt to verify our hypothesis that during puberty the changing environment might have influence on proliferating myocytes. In cases of adverse polymorphisms or mutations in the genes chosen, the cells might respond adversely leading to asymmetrical maturity of the developing muscles.

Two patterns of response to the growth factors were observed: Five genes (CHD7, HDAC5, COL1A1, ACAN, LEF1) were stimulated in their level of expression by IGF-1 with lesser or no effects of FGF2, and one gene (WNT5A) was even down regulated by the addition of FGF2. Only ACTA1 showed an increased expression level that was augmented higher by FGF2 than by IGF-1.

CHD7 belongs to a group of proteins responsible for the organization of chromatin and gene expression and therefore plays a role in regulation of embryonic development. The CHD7 gene itself regulates genetic expression by chromatin remodeling [22]. The group of HDACs, constitutively expressed in myoblasts and myotubes, are important in muscle differentiation and also in chromatin remodeling. HDAC5 especially is part of the muscle differentiation, where it controls differential regulation of the gene expression [23]. Collagens in general are responsible for stabilizing different tissues in the body like cartilage, skin, tendon and bone [24]. The protein encoded by ACAN belongs to the family of proteoglycans and is both part of the Extracellular Matrix (ECM) in cartilage tissue and part of different types of fibroblasts. Its main function is to produce a rigid, deformable gel which is able to resist compression, so it is an important part for the structure of cartilage and the function of different joints [25]. Thus, CHD7, HDAC5, and LEF1 are implicated in proliferation, and COL1A1 and ACAN expression need to increase in proliferating cells. Both FGF2 and IGF-1 induce proliferation of skeletal muscle cells and therefore should have a stimulating influence on these genes, but only the addition of IGF-1 to the cultured cells enhance considerably the expression of these genes. Obviously, FGF2 induces differentiation that might be explained by the recruitment

Discussion

Human myoblast cultures are an appropriate tool to study developmentally critical genes and their effects on muscle development [11]. A better knowledge of their expression patterns and regulation by growth factors will prove useful for insights into the pathomechanisms of diseases associated with mutations in these genes. These data will allow to monitor myocytes obtained from patients suffering from disease originating from genetic defects in these genes. Changes in mRNA levels have been shown to be the primary genetic defects in muscular dystrophies, including mRNA for embryonic myosin heavy chain, α-cardiac actin, versican, acetylcholine receptor α-1, thrombospondin 4 and others [12], and a heterozygous missense mutation in the MEGF10 gene was found to impair the regeneration of adult muscle in response to injury or disease and leads to myopathy and scoliosis [13]. Because the basic

Figure 1: Growth factors and representative expression levels of 5 genes.
Myocytes in vitro culture were treated with Insulin-Like Growth Factor 1 (IGF1) or Fibroblast Growth Factor (FGF). For the Quantitative analysis, we compared expression levels of the genes shown with the expression level of Glyceraldehydes 3-Phosphate Dehydrogenase (GAPDH) (comparative CT method-ΔCT). Error bars represent standard deviation of triplicate qPCR measurements. Expression levels in untreated control cells are normalized to 1 and are thus not shown.

Figure 2: Growth factors and representative expression levels of 2 genes.
Myocytes in vitro culture were treated with Insulin-Like Growth Factor 1 (IGF1) or Fibroblast Growth Factor (FGF). For the Quantitative analysis, we compared expression levels of the genes shown with the expression level of Glyceraldehydes 3-Phosphate Dehydrogenase (GAPDH) (comparative CT method-ΔCT). Error bars represent standard deviation of triplicate qPCR measurements. Expression levels in untreated control cells are normalized to 1 and are thus not shown.
of SHP2 (Src Homology 2 Phosphatase-2) through FGFR activation-induced tyrosine phosphorylation of FRS2 (SNT) (FGFR Stimulated2 Grb2 binding protein), which in turn induces recruitment of GRB2 (Growth Factor Receptor Bound Protein-2), SOS, GAB1 (GRB2 Associated Binding protein-1), and SHP2 (Src Homology 2 Phosphatase-2). Receptor-mediated induction of the SHP2-Ras-ERK pathway is a central, evolutionarily conserved mechanism by which FGFRs elicit a broad spectrum of biological activities, including cell growth, differentiation and morphogenesis [26]. Stimulated differentiation by FGF2 addition is further corroborated by the enhanced expression of ACTA1 as seen in the treated cultured cells. Skeletal alpha-actin belongs to the family of actin proteins, which are necessary for muscle contraction and cell movement, and support in maintaining the cytoskeleton.

LEFI belongs to the high mobility group protein family. This transcription factor participates in the Wnt signaling pathway which is important for embryonic development [27]. The WNT family of secreted glycoproteins are involved in cell proliferation, oncogenesis and several developmental processes, and WNT5A in particular is important during embryogenesis for the development of the primary anterior-posterior axis [17]. Like LEFI, it is part of the WNT signaling pathway. Our data show that—at least in the culture system used-IGF-1 signaling is upstream of LEFI and WNT5 whereas FGF2 has no or even suppressing influence on this pathway.

Conclusion

In summary, we could explicate the feasibility of our myocyte culture system to study genes with possible implication in the development of scoliosis. Growth factor addition to these cells exhibit differential effects simulating eventually the changing growth factor environment during puberty. Any disturbance of the intricate pattern of the various pathways studied might have long lasting effects on skeletal muscle development leading to human disease and might be probed in affected individuals.

References

1. McMaster MJ. Congenital scoliosis. In: Weinstein SL, ed. The pediatric spine: Principles and Practice. Philadelphia, PA: Lippincott Williams & Williams. 2001; 61-177.
2. Offiah A, Alman B, Cornier AS, Giampietro PF, Tassy O, Wade A, Turpenny PD. Pilot assessment of a radiologic classification system for segmentation defects of the vertebral. Am J Med Genet. 2010;152A: 1357-1371.
3. Shahcheraghi GH, Hobi MH. Patterns and progression in congenital scoliosis. J Pediatr Orthop. 1999;19: 766-775.
4. Pourquie O. Segmentation of the vertebrate spine: from clock to scoliosis. Cell. 2011; 145: 650-663.
5. Xiaocong G et al. CHD7. Gene Polymorphisms are associated with Susceptibility to idiopathic scoliosis. Am J Hum Genet. 2007; 80: 957-965.
6. Spencer GS, Zorab PA. Spinal muscle in scoliosis. Part 1. Histology and histochemistry. J Neurol Sci. 1976; 30: 137-142.
7. Bylund P, Jansson E, Dahlberg E, Eriksson E. Muscle fiber types in thoracic erector spinae muscles. Fiber types in idiopathic and other forms of scoliosis. Clin Orthop Relat Res. 1987; 214: 222-228.
8. Ford DM, Baglioni KM, McFadden KD, Greenhill BJ, RasoVj. Paraspinal muscle imbalance in adolescent idiopathic scoliosis. Spine (Phila Pa 1976). 1984; 9: 373-376.
9. Mannion AF, Meier M, Grob D, Müntener M. Paraspinalse muscle fibre type alterations associated with scoliosis: an old problem revisited with new evidence. Eur Spine J. 1998; 7: 289-293.
10. Meier MP, Klein MP, Krebs D, Grob D, Müntener M. Fiber transformations in multifidus muscle of young patients with idiopathic scoliosis. Spine (Phila Pa 1976). 1997; 22: 2357-2364.
11. Abdul-Hussein S, van der Ven P, Tajshargh H. Expression profiles of muscle disease-associated genes and their isoforms during differentiation of cultured human skeletal muscle cells. BMC Musculoskeletal Disorders. 2012; 13: 262.
12. Chen Y-W, Zhao P, Borup R, Hoffman EP. Expression profiling in the Muscular Dystrophies: Identification of new aspects of molecular pathophysiology. J Cell Biol. 2000; 151: 1321-1336.
13. Boyden SE, Mahoney LJ, Kawahara G, Myers JA, Mitsuhashi S, Estrela EA, et al. Mutations in the satellite cell gene MEGF10 cause a recessive congenital myopathy with minicores. Neurogenetics. 2012; 13: 115-124.
14. Fürst DO, Osborn M, Weber K. Myogenensis in the mouse embryo: differential onset of expression of myogenic genes and the involvement of tinif in myofibril assembly. J Cell Biol. 1989; 109: 517-527.
15. Gautel M, Fürst DO, Cocco A, Schiaffino S. Isoforms transitions of the binding protein C family in developing human and mouse muscles: lack of isoform transcomplementation in cardiac muscle. Circ Res. 1998, 82: 124-129.
16. Weist MR, Wellington MS, Bermudez JE, Kostrominova TY, Mendias CL, Arruda EM, et al. TGF-β1 enhances contractility in engineered skeletal muscle. J Tissue Eng Regen Med. 2013; 7: 562-571.
17. Saitoh T, Katoh M. Molecular cloning and characterization of human WNT5B on chromosome 12p13.3 region. Int J Oncol 2001; 19: 347-351.
18. Mitchell P. “Expression of fibroblast growth factor family during postnatal skeletal muscle hypertrophy.” Journal of Applied Physiology. 1998; 86: 313-319.
19. Liu X. “Expression of fibroblast growth factor 2 and its receptor during skeletalmuscle development from turkeys with different growth rates,” Domestic Animal Endocrinology. 2003; 25: 215-229.
20. Adams GR, Haddad F. “The relationships among IGF-1, DNA content, and protein accumulation during skeletal muscle hypertrophy,” Journal of Applied Physiology. 1996; 81: 2509-2516.
21. Takano K, Watanabe-Takano H, Suetsugu S, Kurita S, Tsujioka K, Kimura S, et al., “Nebulin and N-WASP Cooperate to Cause IGF-1-Induced Sarcomeric Actin Filament Formation,” Science. 2010; 330: 1536-1540.
22. Kools P, Van Imschoot G, van Roy F. Characterization of three novel human cadherin genes (CDH7, CDH19, and CDH20) clustered on chromosome 18q22-q23 and with high homology to chicken cadherin-7. Genomics. 2000; 68: 283-295.
23. Mahlknecht U. “Chromosomal organization and localization of the human histone deacetylase 5 gene (HDAC5),” Biochimica et Biophysica Acta. 2000; 1493: 342-348.
24. Val Mann, Emma E Hobson, Bachua Li, Tracy L Stewart, Straun FA Grant, Simon P Robins, et al “A COL1A1 Sp1 binding site polymorphism predisposes to osteoporotic fracture by affecting bone density and quality,” The Journal of Clinical Investigation. 2001; 107: 899-907.
25. Roughley P, Martens D, Rantakokko J, Alini M, wale FM, Antoniou J et al., “The involvement of aggrecan polymorphism in degeneration of human intervertebral disc and articular cartilage,” European Cells and Materials. 2006; 11: 1-7.
26. Nusse R. “Wnt signaling in disease and in development,” Cell Research. 2005; 15: 28-32.
27. Milatovich A, Travis A, Grosschedl R, Francke U. “Gene for lymphoid enhancer-binding factor 1 (LEFI) mapped to human chromosome 4 (q23-q25) and mouse chromosome 3 near Egf.” Genomics. 1991; 18q22-q23 and with high homology to chicken cadherin-7. Genomics. 2000; 151: 1321-1336.

Citation: Huber K, Kraupa C, Kluger R and Krugluker W. Gene Expression Profiles Induced by Growth Factors in In Vitro Cultured Myocytes. SM Musculoskelet Disord. 2018; 3(2): 1031. https://dx.doi.org/10.36876/smmd.1031